Clinical and Experimental Studies on Nephrin

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I. Holthöfer H, Ahola H, Solin ML, Wang SX, Palmén T, Luimula P, Miettinen A, Kerjaschki D (1999). Nephrin localizes at the podocyte filtration slit area and is characteristically spliced in the human kidney. *Am J Pathol* 155:1681-1687

II. Wang SX, Menè P, Holthöfer H. Nephrin mRNA regulation by protein kinase C. *J Nephrol* (in press)

III. Wang SX, Ahola H, Palmén T, Solin ML, Luimula P, Holthöfer H (2001). Recurrence after transplantation in CNF is due to autoantibodies to nephrin. *Exp Nephrol* (in press)

IV. Wang SX, Rastaldi MP, Ahola H, Heikkilä E, Holthöfer H. Patterns of nephrin and a new proteinuria-associated protein expression in human renal diseases. *Kidney Int* (submitted)

ABBREVIATIONS

aa	amino acid
$[Ca^{2+}]_i$	intracellular calcium
CD2AP	CD2-associated protein
cDNA	complementary DNA
CNF	congenital nephrotic syndrome of the Finnish type
cPKC	classic PKC
C-terminus	carboxyterminus
ELISA	enzyme-linked immunoadsorbent assay
EM	electron microscopy
FCS	fetal calf serum
FSGS	focal segmental glomerulosclerosis
GBM	glomerular basement membrane
HSPG	heparan sulfate proteoglycan
IF	immunofluorescence
kb	kilobase
kDa	kilodalton
mAb	monoclonal antibody
MCGN	minimal change glomerulonephropathy
MGN	membranous glomerulonephropathy
MPGN	membranoproliferative glomerulonephritis
M_r	relative molecular weight
mRNA	messenger RNA
MsPGN	mesangial proliferative glomerulonephritis
NPHS1	gene for nephrin
nPKC	novel PKC
N-terminus	aminoterminus
pAb	polyclonal antibody
PAGE	polyacrylamide gel electrophoresis
PAN	puromycin aminonucleoside necrosis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKC	protein kinase C
PMA	phorbol-12-myristate-13-acetate
ROS	reactive oxygen species
RT	reverse transcription
SDS	sodium dodecyl sulphate
TGF	transforming growth factor
TJ	tight junction
ZO	zonula occludens

SUMMARY

Nephrotic syndrome is a clinical entity with multiple kinds of causes. It is characterized mainly by increased glomerular permeability manifested by massive proteinuria, in addition to the tendency towards edema, hypoalbuminemia, and hyperlipidemia. Congenital nephrotic syndrome of the Finnish type (CNF) is a specific type of nephrotic syndrome discovered primarily in Finland. The pathogenesis of CNF has remained unknown for decades. In 1998, Kestilä *et al.* reported the mutations of NPHS1, the gene coding for nephrin, in CNF patients. Northern blotting results showed that nephrin is expressed only in the kidney. In accordance with its primary structure, nephrin was predicted to be a putative transmembrane protein with a calculated M_r of 135 kDa. The aims of this study were: (1) to localize nephrin molecules in the glomerulus; (2) to search for the molecules regulating the expression of nephrin within A293 cells; (3) to explore the mechanisms for recurrence of nephrotic syndrome in CNF patients with kidney transplantation; and (4) to observe the expression patterns of nephrin and 18C7 antigen in human renal diseases.

In article I, both immunofluorescence (IF) and immuno-electron microscopy (EM) were used to localize nephrin in normal human kidney. The results showed that nephrin molecules are mainly situated at the slit diaphragm areas. When nephrin mRNA expression was studied by RT-PCR, a higher dominant band and a lower one (designated nephrin-α) were found. Nephrin-α lacks the transmembrane part as compared to the dominant one. In article II, intracellular molecules responsible for the regulation of nephrin expression were sought in A293 cells. The changes in the expression of nephrin were found both at the mRNA and protein level after stimulation of protein kinase C (PKC). Furthermore, these changes were independent of intracellular calcium ($[Ca^{2+}]_i$) concentration variations and were confirmed in both normal human kidney and CNF patient kidney epithelial cells. In article III, enzyme–linked immunoadsorbent assay (ELISA) was used to explore whether autoantibodies to nephrin were formed in transplanted CNF patients with recurrence of nephrotic syndrome. Before recurrence, the autoantibody titer was low, whereas in patients with recurrence of nephrotic syndrome, anti-nephrin antibodies increased. After successful treatment, anti-nephrin antibody levels steadily decreased. Thereby, anti-nephrin antibodies appear responsible for the recurrence of nephrotic syndrome in these CNF patients. Finally, in article IV, the previous study was expanded to non-CNF kidney diseases. One hundred and twenty kidney biopsy samples were used to search for changes in nephrin expression by immunohistochemistry. The results did not demonstrate significant differences among different diagnostic groups. A monoclonal antibody (mAb) 18C7 was also used to screen these kidney samples. It was found that the thickness of the glomerular basement membrane (GBM) correlated with 18C7 staining intensity. Therefore, nephrin was

thought to be not the only molecule relevant to proteinuria. It is possible that the 18C7 protein molecule also participates in the regulation of the glomerular filtration barrier.

The findings of this study are certainly helpful in delineating the complicated mechanisms of glomerular proteinuria. Nephrin was the first transmembrane protein molecule reported at the slit diaphragm and this together with its discovery in A293 cells, in turn, will stimulate further studies in signal transduction and cellular junctions both *in vitro* and *in vivo*. The existence of autoantibodies to nephrin should prompt a search for more effective methods of treatment in CNF patients with recurrence of nephrotic syndrome. The differences of expression patterns of 18C7 antigen in distinct human renal diseases suggest the complexity of glomerular proteinuria and the necessity for searching for more proteinuria-associated molecules.

INTRODUCTION

Glomerular proteinuria is very important in indicating glomerular injury. Glomerular proteinuria is due to either changes of the biochemical and the permselective properties of the glomerular capillary wall or hemodynamic factors. Though the landmark experiment 35 years ago provided direct evidence to show that GBM is the main filtration barrier to proteins (Farquhar *et al.* 1961), there was also evidence that the ultimate barrier for proteins of the size of albumin resides in the slit diaphragm (Karnovsky and Ainsworth 1972, Tryggvason 1999).

CNF is an autosomal recessive disease, belonging to the Finnish disease heritage. For decades, its pathogenesis has remained unknown. Genes coding for the main components of GBM have been ruled out in CNF patients (Kestilä *et al.* 1994b, Lenkkeri 1998). In 1998, Kestilä *et al.* (1998) successfully cloned NPHS1. NPHS1, containing totally 29 exons, was assigned to chromosome 19q13.1 (Kestilä *et al.* 1998, Lenkkeri *et al.* 1999). The deletion (CT) in exon 2 (Fin_{major}) and a nonsense mutation ($C \rightarrow T$) in exon 26 (Fin_{minor}) both lead to the heavy proteinuria seen in CNF patients. It has been reported that over 90% of CNF patients (Fin_{major} and Fin_{minor}) have defective expression of nephrin (Kestilä *et al.* 1998, Lenkkeri *et al.* 1999). Hybridisation *in situ* showed that within the kidney, nephrin is exclusively expressed in the glomerular visceral epithelial cells, the podocytes (Kestilä *et al.* 1998). Nephrin, the protein product of NPHS1, is a putative transmembrane protein with 1,241 residues and a calculated M_r of 135 kDa without posttranslational modifications (Kestilä *et al.* 1998).

The exact location of nephrin in the glomerulus and the significance of nephrin in CNF patients with recurrence of nephrotic syndrome after kidney transplantation, as well as the expression of nephrin in kidneys of non-CNF human renal diseases have not been clear. Therefore, in this thesis, we have focused on these issues. Furthermore, the intracellular regulators responsible for nephrin have been searched for in A293 cells, a cell line derived from human embryonic kidney cells. Additionally, mAb 18C7, a monoclonal antibody associated with proteinuria, was also used to screen the kidney tissue sections of these non-CNF human renal diseases.

REVIEW OF THE LITERATURE

1. Structure and function of the kidney

The two kidneys are a pair of bean-shaped organs situated in the retroperitoneal space. Generally, the kidney size of the adult human averages 11-12 cm in length, 5.0-7.5 cm in width and 2.5-3.0 cm in thickness. The weight of each kidney varies from 125 to 170 g in the adult male and 115 to 155 g in the adult female (Tisher and Madsen 1991). The kidney is successively covered by three layers (from the interior to the exterior): fibrous capsula, adiposa capsula and renal fascia (Ling 1990). On the surface of a bisected kidney, two distinct regions can be identified. The outer cortex part, rich in glomeruli, proximal tubules and some of distal tubules, appears pale; the inner medulla part, rich in Henle loops and collecting ducts, looks darker (Tisher and Madsen 1991). The structural and functional unit of the kidney is nephron. It is estimated that every kidney has about one million nephrons (Zou 1997). Every nephron consists of one renal corpuscle and its associated tubules. Each renal corpuscle is further divided into glomerulus, Bowman's capsule and juxtaglomerular apparatus. One glomerulus is composed of 5-7 capillary branches originating from the afferent artery, whose surface is extensively covered by glomerular podocytes. The lumen of the glomerular capillary is lined by a thin fenestrated endothelium. Between the podocytes and endothelium is a layer of a mesh-like structure, the glomerular basement membrane, which usually is thought to be the principal structure in preventing leakage of plasma macromolecules (**Fig. 1**). Each kidney is supplied by a single renal artery arising from the descending aorta. The renal nerve, belonging to the autonomic nervous system, arises mainly from the celiac plexus, with additional contributions from the greater splanchnic nerve, the superior hypogastric plexus, and the intermesenteric plexus (Tisher *et al.* 1991, Tisher and Madsen 1991).

The kidney has physiologically multiple functions. It is vital for maintaining the stable homeostasis of the human body. The kidney continuously excretes different kinds of metabolic wastes, such as creatinine, urea, uric acid, and foreign substances; it also regulates body fluid, osmolarity, electrolytes such as sodium and potassium, as well as acid-base balance via its extremely effective functions of concentration and dilution, reabsorption and secretion. Furthermore, the kidney can produce and secrete erythropoietin and then regulate the maturation process of erythrocytes in the bone marrow. The kidney can also secrete renin, generally stored in juxtaglomerular cells and extraglomerular mesangial cells. Renin can be used to adjust blood volume, blood vessel contraction and promote the secretion of other hormones. The kidney is a key site for the activation of 1,25-dihydroxyvitamine D_3 (Tisher and Madsen 1991, Zou 1997).

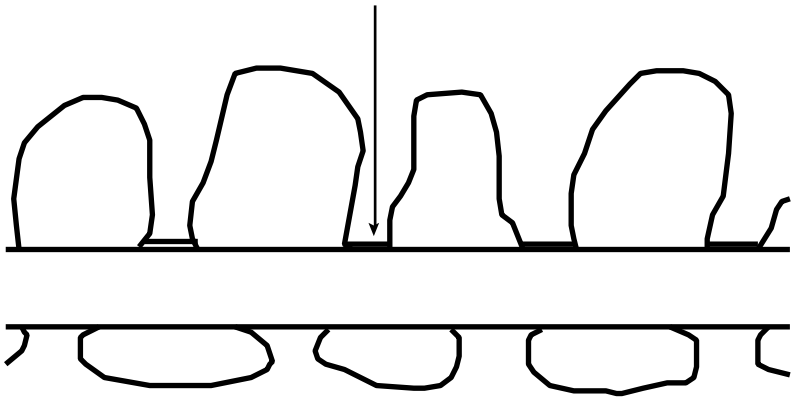

Figure 1. Structure of the glomerular filter: endothelium, GBM, and epithelium

2. Glomerular podocytes

Podocytes, forming the outmost layer of the glomerular filter, play several significant roles. They maintain the stability of the glomerular structure by counteracting capillary wall distensions (Kriz *et al.* 1994, Kriz *et al.* 1995, Kriz *et al.* 1996); they constitute a relatively large filtration surface to augment the filtration function (Drumond *et al.* 1994, Pavenstädt 2000); and perhaps the most important is the highly specialized and interdigitating foot processes making up the zipper-like structure, the slit diaphragm. So far, multiple kinds of podocyte-specific and/or important molecules have been found. Of these, some may play comparatively significant roles in maintaining the shape of podocytes and the integrative structure of the slit diaphragm, while some others may contribute more to the communication either "outside-in" or "inside-out" between the foot processes of podocytes and to the signalling from cell membrane to the nucleus and *vice versa*.

2.1. Structure and function of the podocyte-specific and/or -important molecules

Tissue- or cell-specific molecules are important in determining the special functions of the cells. The following is a discussion of thirteen podocyte-specific and/or -important molecules.

Nephrin

Nephrin was originally identified by Kestilä *et al.* (1998). With linkage analysis, NPHS1, the gene for nephrin, which contains totally 29 exons, was assigned to chromosome 19q13.1 (Lenkkeri *et al.* 1999). The deletion (CT) in exon 2 (Fin_{major}), causing a frameshift and translation stop in the same exon, and a nonsense mutation (C→T) in exon 26 (Fin_{minor}) both

lead to the massive proteinuria and edema seen in CNF patients. Fin_{major} and Fin_{minor} were confirmed in over 90% of CNF patients (Kestilä *et al.* 1998), suggesting that mutations of NPHS1 are responsible for the CNF disease. Nephrin is a putative transmembrane protein with 1,241 residues and a calculated M_r of 135 kDa without posttranslational modifications. Nothern blotting and *in situ* hybridization showed that nephrin is expressed uniquely in the glomerular visceral epithelial cells, the podocytes (Kestilä *et al.* 1998). Following the report of NPHS1, the homologues of rat and mouse have been successively cloned (Ahola *et al.* 1999, Holzman *et al.* 1999, Kawachi *et al.* 2000, Putaala *et al.* 2000). The analysis of amino acids showed that mouse and rat share about 82% identity with the overlapping sequences of human nephrin and 93% identity with each other (Ahola *et al.* 1999, Putaala *et al.* 2000). In addition, nephrin was also identified in *C. elegans* (Teichmann and Chothia 2000).

Before nephrin was cloned, Orikasa *et al.* (1988) reported that mAb 5-1-6 could recognize one rat antigen, termed p51. p51 was found to be expressed exclusively on the basolateral plasma membrane areas of developing visceral epithelial cells in the S-shaped body (Kawachi *et al.* 1995, Topham *et al.* 1999). Recently, double staining was used to demonstrate the colocalization of nephrin and p51. mAb 5-1-6 was also found to interact with the extracellular part of nephrin by immunoprecipitation and Western blotting (Topham *et al.* 1999). Subsequently, Topham *et al.* (1999) used mass spectrometry to explore the relationship between nephrin and p51. Two peptides cleaved from p51 showed identity with amino acids of nephrin. Finally, it was concluded that p51, the mAb 5-1-6-recognized antigen, is rat nephrin (Kawachi *et al.* 2000).

P-cadherin

P-cadherin belongs to a large cadherin family with more than 40 members (Lodish *et al.* 1999). For the first time, Tassin *et al.* (1994) found the presence of P-cadherin during *in vivo* maturation of renal podocytes. Afterwards, P-cadherin was further confirmed in the ureteric buds and in the upper limb of S-shaped bodies (Goto *et al.* 1998). Very recently, Reiser *et al.* (2000) found the co-expression of P-cadherin with zonula occludens-1 (ZO-1) in cultured podocyte cells and *in vivo* slit diaphragm.

Podocin

NPHS2, a causative gene for autosomal recessive steroid-resistant nephrotic syndrome, was previously mapped to 1q25-q31 and very recently cloned (Fuchshuber *et al.* 1995, Boute *et al.* 2000). The complete open reading frame of NPHS2 is 1,149 bp long and the deduced 383 amino acids from full-length cDNA encodes a putative protein, podocin, whose M_r is of approximately 42 kDa (Boute *et al.* 2000). Structural analysis of the podocin suggested that it is an integral membrane protein with one transmembrane domain (aa 105-121) and a 262 aa C-terminal cytoplasmic part. Podocin belongs to the stomatin protein family, whose one character is to form homo-oligomeric complexes via its C-terminus (Snyers *et al.* 1998). *In situ*

hybridization indicated that NPHS2 is almost exclusively expressed in podocytes of fetal and mature kidney glomeruli but not in other tissues. Dot blot provided further results. The kidney dot showed a strong signal, while several other tissues such as adult testis, fetal heart and liver indicated weak ones (Boute *et al.* 2000). Gene mutation analysis found that the mutations mainly include nonsense, frameshift, and missense (Boute *et al.* 2000). Subcellular localization of podocin is not yet known, but it was proposed to play different roles by forming a widespread structure of homo-oligomers in the same way as stomatin (Boute *et al.* 2000, Somlo and Mundel 2000). It may also interact with other podocyte proteins such as nephrin either directly or indirectly, and function as a bridge linking the plasma membrane proteins to the cytoskeleton (Boute *et al.* 2000). NPHS2 seems to be the first identified gene involved in familial focal segmental glomerulosclerosis (FSGS). Regardless of its expression early during kidney development in the metanephros, inactivation of NPHS2 didn't cause a congenital nephrotic syndrome (Boute *et al.* 2000).

Podoplanin

Podoplanin was originally identified as a 43 kDa glycoprotein, and it was localized diffusely on the surface of rat podocytes, the parietal epithelial cells of Bowman's capsule, and many other extrarenal tissues (Breiteneder-Geleff *et al.* 1997). This integral membrane protein is composed of a mucin-like ectodomain containing six potential *O*-glycosylation sites, a transmembrane domain and an intracellular domain with two possible serine phosphorylation sites. Molecular cloning showed that the open reading frame of podoplanin is 498 bp long, and correspondingly coding for 166 amino acids (Breiteneder-Geleff *et al.* 1997). Similar glycoproteins with extensive sequence identities were previously found in other tissues such as rat lung, fetal kidney cortex and brain (Rishi *et al.* 1995), rat and mouse osteoblasts (Nose *et al.* 1990a, Wetterwald *et al.* 1996), mouse thymus epithelium (Farr *et al.* 1992), and lymphatic endothelium (Breiteneder-Geleff *et al.* 1999). Podoplanin was reported to be downregulated by 70% in a puromycin aminonucleoside necrosis (PAN) model both at the protein level and mRNA level (Breiteneder-Geleff *et al.* 1997). Injection of anti-podoplanin polyclonal antibodies (pAb) caused selective binding of IgG to the whole podocyte surface, leading to transient proteinuria and concomitantly a retraction of podocyte foot processes (Matsui *et al.* 1998, Matsui *et al.* 1999). However, proteinuria could not be prevented by complement depletion or pretreatment with the scavenger dimethylthiourea of oxygen radical products (Matsui *et al.* 1999).

Podocalyxin

140 kDa podocalyxin was originally identified by Kerjaschki *et al.* (1984). Following this discovery, the podocalyxin-like proteins have been successively cloned from rabbit (Kershaw *et al.* 1995), chicken (McNagny *et al.* 1997), and human (Kershaw *et al.* 1997, Sassetti *et al.* 1998). All of these proteins share a high homology in the intracellular and

transmembrane domains, while the ectodomain is more heterogeneous by only preserving the mucin-like structure and four conserved cysteines (Miettinen *et al.* 1999). Podocalyxin is a highly glycosylated integral membrane protein with *N*- and *O*-linked carbohydrates, both of which are sialylated and sulfated (Dekan *et al.* 1991), which could contribute to the maintenance of the negative charge in glomerular filter and thus keep the urinary filtration pores open (Miettinen *et al.* 1990). Podocalyxin was found to be mainly distributed on the surface of glomerular podocytes away from slit diaphragm and endothelial cells in the rat (Kerjaschki *et al.* 1984, Horvat *et al.* 1986, Dekan *et al.* 1990, Miettinen *et al.* 1990). In the developing kidney, as early as the S-shaped body stage, podocalyxin was detected in differentiated podocytes and endothelium (Schnabel *et al.* 1989). Recently, Miettinen *et al.* (1999) reported podocalyxin also in rat platelets and megakaryocytes, suggesting its role in hematopoiesis as previously showed in the chicken (McNagny *et al.* 1997).

GLEPP1

GLEPP1 was cloned and characterized primarily in the rabbit (Thomas *et al.* 1994). Subsequently, GLEPP1 was also identified in the human and mouse (Wiggins *et al.* 1995, Wang *et al.* 2000). Nucleotide sequencing comparison showed that human and mouse GLEPP1 are approximately 90% and 80% identical to rabbit; while deduced amino acids analysis indicated higher identity, 97% and 91% (Wiggins *et al.* 1995, Wang *et al.* 2000). The gene for GLEPP1 in the human was assigned to 12p12-p13 by fluorescence *in situ* hybridization (Wiggins *et al.* 1995). GLEPP1 is a receptor-like glycoprotein belonging to protein-tyrosine phosphatase family with a large ectodomain containing multiple fibronectin type III repeats, a transmembrane part, and a single cytoplasmic phosphatase sequence. The rabbit GLEPP1 protein is 1,158 amino acids long and has a calculated M_r of 132 kDa. Nonetheless the actual M_r on the gel is much higher (≈300 kDa) than expected, possibly due to its posttranslational modification *e.g.* glycosylation, as the extracellular domain of GLEPP1 contains 15 potential *N*-linked glycosylation sites (Thomas *et al.* 1994). In a RNase protection assay it was showed that GLEPP1 is distributed in the foot processes of the podocytes and also in the brain, whilst other tissues such as liver, lung, skin, eye, skeletal muscle, placenta, heart, spleen, stomach, small intestine and large intestine are negative (Thomas *et al.* 1994). In developing mouse kidney, Northern blotting identified a single 5.5 kb transcript in fetal kidney that became approximately three-fold more abundant in adults. *In situ* hybridization revealed the existence of GLEPP1 in comma- and S-shaped stages of the visceral epithelial cells, and expression increase in capillary loop and maturing stage glomeruli (Wang *et al.* 2000). In an anti-GBM rabbit model and in patients with crescentic nephritis, GLEPP1 was found to be decreased (Yang *et al.* 1996). In minimal change glomerulonephropathy (MCGN), GLEPP1 was shifted away from the GBM on the apical cell membrane of effaced foot processes. In FSGS, glomerular GLEPP1 was often absent from the podocytes (Sharif *et al.* 1998). It was speculated that the homophilic and/or

heterophilic interactions between ectodomains of GLEPP1, especially fibronectin III, could be involved in cell-cell and/or cell-matrix interactions (Thomas *et al.* 1994, Wang *et al.* 2000).

Megalin

Megalin (gp600) was identified originally by Kerjaschki and Farquhar (1982). The deduced amino acids revealed that its M_r in a glycosylated form is around 600 kDa. Megalin is one of the largest eucaryotic glycoproteins (Makker and Singh 1984, Saito *et al.* 1994). Megalin is composed of a larger ectocellular domain (4,400 aa residues), a single transmembrane section (22 residues) and a short cytoplasmic part (213 residues). All the present information on structure and localization of megalin suggests it is an anchored receptor for mediating endocytosis, belonging to the low density lipoprotein receptor superfamily (Kerjaschki and Farquhar 1983, Kerjaschki *et al.* 1987, Kerjaschki and Neale 1996, Kerjaschki *et al.* 1997). Human megalin homologue gene was located on chromosome 2q24-q31. Megalin was initially localized on the podocytes, and was expressed in clathrin-coated pits on the bases of foot processes of podocytes and proximal tubular cells (Kerjaschki and Farquhar 1982, Makker and Singh 1984). In addition, megalin was also found to be present in other tissues such as lung, thyroid, parathyroid, ependyma, ciliary epithelium in adult and trophectoderm, neuroectoderm in embryo (Zheng *et al.* 1994). An analysis of megalin-deficient mice showed an abnormal formation of the forebrain and forebrain-derived structures as well as changed ultrastructure of proximal tubules (Willnow *et al.* 1996, Christensen and Willnow 1999).

Integrin

Integrins are a group of dimeric proteins that contain both α- and β-subunits. At the present time, 17 α- and 8 β-subunits have been identified in mammals, totally comprising 23 dimers (Humphries 2000). The integrin in podocytes is mainly α3β1, distributed on the bases of the foot processes along GBM (Kreidberg and Symons 2000). α3β1 in podocytes has many ligands, such as collagen, fibronectin, laminin-11, entactin/nidogen, and epiligrin (Adler and Brady 1999). The importance of integrin has been addressed by the knockout mice (Kreidberg *et al.* 1996). It was found that podocytes deficient in integrin (α3β1) seemed unable to form the mature foot processes of the glomeruli; the cell body of podocytes was flattened against GBM; the GBM was not continuous but fractionated; few capillary loops of the glomeruli were observed. In membranous glomerulonephropathy (MGN), decreased staining of α3β1 integrin was found (Shikata *et al.* 1995).

Synaptopodin

Synaptopodin, also previously named "pp44", was reported to be a novel actin-associated cytoplasmic protein. The open reading frame of synaptopodin encodes a 685/690 aa polypeptide (human/mouse) with a calculated M_r 73.7/74.0 kDa (Mundel *et al.* 1997). However, the actual M_r of synaptopodin determined by SDS-PAGE and Western blotting is about 100 kDa, which was suspected to be due to either posttranslational modifications or conformational

changes (Mundel *et al.* 1997). During developmental stages of the kidney, synaptopodin started to appear at the capillary loop stage (Mundel *et al.* 1991). Synaptopodin was also expressed in foot processes of the mature podocytes and brain (Mundel *et al.* 1997). Hence, synaptopodin was thought to be one of the maturation markers in the podocytes. In collapsing idiopathic focal glomerulosclerosis, synaptopodin expression seemed to be downregulated together with GLEPP1 and podocalyxin (Barisoni *et al.* 1999). Synaptopodin even disappeared in the areas of capillary wall necrosis, cellular crescents, or at early and advanced stages of FSGS (Kemeny *et al.* 1997).

CD2AP

CD2-associated protein (CD2AP)/CMS (human) was identified firstly in a yeast two-hybrid system (Dustin *et al.* 1998, Kirsch *et al.* 1999). Mouse CD2AP is an adapter protein interacting with the cytoplasmic domain of CD2, increasing CD2 clustering and cytoskeletal polarization. The deduced number of amino acids from full-length cDNA is approximately 641 with a calculated M_r 70 kDa (Dustin *et al.* 1998). Interestingly, CD2AP was recognized as a 80 kDa protein by Western blotting (Dustin *et al.* 1998, Lehtonen *et al.* 2000). IF findings revealed that CD2AP is widely expressed (Lehtonen *et al.* 2000). In the kidney, CD2AP was located not only at glomerular podocytes, but weakly at tubular epithelial cells (Lehtonen *et al.* 2000). CD2AP-deficient mice exhibited a similar phenotype with CNF patients (Shih *et al.* 1999), indicating its crucial role in the maintenance of the glomerular filtration barrier (Somlo and Mundel 2000). CD2AP was found to interact directly with polycystin-2 and $P130^{Cas}$ in the yeast two-hybrid system. This was also found to be true in *in vitro* cell culture by coimmunoprecipitation. Double staining showed the colocalization of CD2AP and polycystin-2. These data further provided insight into the function of CD2AP in mature renal tubular epithelium (Lehtonen *et al.* 2000).

ZO-1

A tight junction (TJ)-enriched membrane fraction was used as immunogen to generate mAb specific for this intercellular junction. One antigen, termed ZO-1, was found to be concentrated at the junctional complexes of colon, kidney, and testis (Stevenson *et al.* 1986). ZO-1 is the first identified TJ protein with a M_r of 210-225 kDa on SDS-PAGE (Stevenson *et al.* 1986). However, ZO-1 was also detected in the nucleus (Gottardi *et al.* 1996). Molecular cloning showed that the deduced amino acids are 1,736 and the calculated M_r is 195 kDa (Willott *et al.* 1993). The discrepancy of M_r was thought to contribute to the proline-rich of ZO-1 (Willott *et al.* 1993). Physical mapping has indicated its locus at 15q13 in human (Mohandas *et al.* 1995). There exist two isotypes of ZO-1, as a result of alternative RNA splicing, differing by 80 aa domain (Willott *et al.* 1992). ZO-1 belongs to a membrane-associated guanylate kinase protein family, which share structural similarities, including one to three PDZ (PSD-95/SAP90, Dlg, ZO-1) domains, an SH3 domain, and a region of homology with the enzyme guanylate

kinase (Mitic and Anderson 1998). Protein ZO-1 was found to be concentrated along the slit diaphragm of the glomerular epithelium in newborn and adult rat kidneys (Schnabel *et al.* 1990, Macconi *et al.* 2000). Injection of mAb 5-1-6 reduced the expression of ZO-1 in rat podocytes, in accompany with heavy proteinuria (Kawachi *et al.* 1997). All these suggest the function of ZO-1 in preventing the leakage of plasma proteins.

α-actinin-4

α-actinin-4, encoded by its corresponding gene ACTN4, was found to be the actin-binding and crosslinking cytoplasmic protein. Expression of ACTN2 and ACTN3 is limited to the skeletal and cardiac muscle sarcomere, while ACTN4 and ACTN1 is widely expressed (Honda *et al.* 1998, Kaplan *et al.* 2000). In the glomerulus, ACTN4 was showed to be located predominantly in the foot processes (Smoyer *et al.* 1997). Kaplan *et al.* (2000) reported the mutations in ACTN4 from familial FSGS patients, suggesting the potential role of α-actinin-4 in regulation of the actin cytoskeleton of glomerular podocytes.

WT1

Wilms' tumor specific gene, WT1 gene located in 11p13, has been cloned and characterized (Call *et al.* 1990, Gessler *et al.* 1990). The WT1 gene contains totally ten exons encoding a zinc finger protein, occupying approximately 50 kb of genomic DNA (Salomon *et al.* 2000). Exons 1-6 encode a transcriptional regulatory region rich in proline/glutamine, exons 7-10 encode DNA binding domain with four zinc finger motifs (Call *et al.* 1990, Gessler *et al.* 1992). There are totally four splicing variants, due to two splicing regions of exon 5 and exon 9 by the 3' end (Haber *et al.* 1991). The sublocalization of WT1 protein was defined exclusively as nuclear by using confocal laser microscopy (Mundlos *et al.* 1993). The WT1 protein has several features of a transcription factor, including four Cys2-His2 zinc finger motifs at the C-terminus (Call *et al.* 1990), moreover, its four isotypes have distinct subnuclear locations and play roles in posttranscriptional processing of RNA as well as in transcription (Englert *et al.* 1995, Larsson *et al.* 1995, Caricasole *et al.* 1996). As a transcription factor, it has several target genes, such as insulin-like growth factor 2 (Drummond *et al.* 1992), platelet-derived growth factor A-chain (Wang *et al.* 1992), transforming growth factor (TGF)-β1 (Dey *et al.* 1994), paired-box (PAX2) and PAX8 (Yang *et al.* 1999), and nov (Martinerie *et al.* 1996). *In situ* hybridization showed that WT1 is selectively expressed in metanephric blastema, S-shaped bodies and glomerular epithelium during kidney development, and also found to be expressed in human undifferentiated gonadal ridge (Pritchard-Jones *et al.* 1990), and glomerular podocytes and parietal epithelial cells at Bowman's capsule in adulthood by RT-PCR (Mundlos *et al.* 1993). Knockout mice of WT1 gene resulted in the absence of both kidneys and gonads, suggesting a crucial role in the development of the genitourinary tract. WT1 plays a major part in the induction of the ureteric bud, the mesenchymal to epithelial differentiation, the progression

of nephrogenesis, and the maintenance of podocyte functions. Denys-Drash syndrome and Frasier syndrome are diseases caused by WT1 gene mutations (Salomon *et al.* 2000).

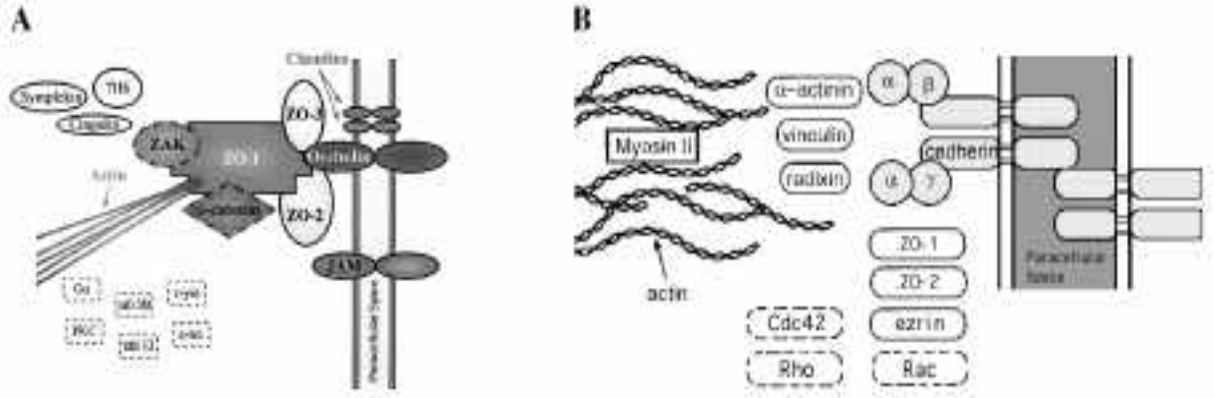

Figure 2. Molecular architecture of the tight junction (A) and adherents junction (B). α, α-catenin; β, β-catenin; γ, γ-catenin (modified from Fanning *et al.* 1999, Mitic and Anderson 1998)

2.2. Slit diaphragm and molecular architecture of the cellular junctions

Generally, there exist four types of cellular junctions, *i.e.*, occluding or tight junctions, adherents junctions, gap junctions, and desmosomes (Gumbiner 1996). All of them are mainly composed of three parts, *i.e.*, cell receptors, intracellular plaque or peripheral membrane proteins, and extracellular ligands. The cell receptor should be the key molecule, as it appears to determine the type of cellular junction.

The tight junction (**Fig. 2, A**) is usually the most apical component of the junctional complexes separating the apical and basolateral plasma membrane to generate and maintain cell polarity, and functioning as a paracellular barrier to selectively prevent some molecules passing freely (Denker and Nigam 1998, Cereijido *et al.* 1998, Tsukita *et al.* 1999, Tsukita and Furuse 2000). Though two models ("protein" model and "lipid" model) are used to explain the structure and function of TJs (Tsukita *et al.* 1999), most identified molecules so far support the former one. It is indicated that TJs are closely associated with at least nine integral or peripheral protein molecules (Stevenson and Keon 1998). Out of these, occludin and claudin seem the key molecules (Furuse *et al.* 1993, Furuse *et al.* 1998). In addition, JAM (a junction-associated membrane protein), ZO (1, 2, and 3), cingulin, 7H6 antigen, ZAK, symplekin also have been localized in TJs (Stevenson and Keon 1998, Tsukita *et al.* 1999, Fanning *et al.* 1999).

The adherents junctions (**Fig. 2, B**) are another type of specialized cellular junctions based mainly upon cadherin molecules as the core across the plasma membrane (Yap *et al.* 1997). So far, over 40 members of cadherins have been reported (Lodish *et al.* 1999). E-, P-, N-, and R-cadherins are the commonest. It has been suggested that the formation of homodimers of

cadherins is the basic unit for the adhesive function (Shapiro *et al.* 1995). The homophilic interaction site has been localized to the first extracellular domain of N-terminus (EC1, Nose *et al.* 1990b). Nonetheless, this is not universal, N- and R-cadherins can interact with each other nearly at the same site (Yap *et al.* 1997), raising the possibility that they could form heterodimers if they both existed at the same location. Furthermore, many adherents junction-associated proteins have been presented, such as α-, β-, δ-catenin, plakoglobin (γ-catenin), vinculin, ezrin, moesin, radixin, p120, ZO-1, ZO-2, afadin, and shroom (Yap *et al.* 1997, Troyanovsky 1999, Gumbiner 2000, Critchley 2000, Anastasiadis and Reynolds 2000). The mechanism of adherents junction assembly has already been extensively explored (Kusumi *et al.* 1999, Troyanovsky 1999). Rho small GTPase family (Rac1, Rho, Cdc42) play important roles by regulating cadherin-mediated cell-cell adhesion (Kaibuchi *et al.* 1999). A wide range of extracellular signals, including growth factors, gap junction-associated communication, peptide hormones, and cholinergic receptor agonists, influence the adherents junction (Yap *et al.* 1997). Beyond basic adhesive function, adherents junctions also have a series of broad functions, including tissue formation and stability, cell migration, and regulation of intracellular signaling events (Yap *et al.* 1997, Steinberg and McNutt 1999).

The third cellular junction is gap junction, which is deeply involved in many functions of the cell, such as proliferation, differentiation, and apoptosis, and also involved in the exchange of solutes, metabolic precursors and electrical currents between neighbouring cells (Eggleston 2000). The main constituents for gap junction are the connexins, which are the subunits of the connexons (Trosko *et al.* 2000).

Desmogleins and desmocollins, the cell adhesion receptors, which are indirectly linked to the intermediate filament by desmoplakins and plakoglobin, are the key components in assemblying the desmosomal junction (Gumbiner 1996).

Two decades ago, Rodewald and Karnovsky (1974) reported that under EM the slit diaphragm was a zipper-like structure with the slit in the middle. Subsequently, more efforts have been made towards its structural analysis. However, little progress has been made. One probable reason is, due to the resolution limits of EM, even today the slit diaphragm appears just as a line under the most advanced EM. Another reason is the lack of appropriate candidate molecules responsible for the assembly of the slit diaphragm during embryo development. With the cloning of nephrin gene NPHS1, hopefully, the structure of the slit diaphragm can be analyzed in detail during the coming years. Indeed, it is true that the charaterization of the slit diaphragm has advanced recently with the new discovery of slit diaphragm-related proteins, such as P-cadherin and CD2AP (**Fig. 3**).

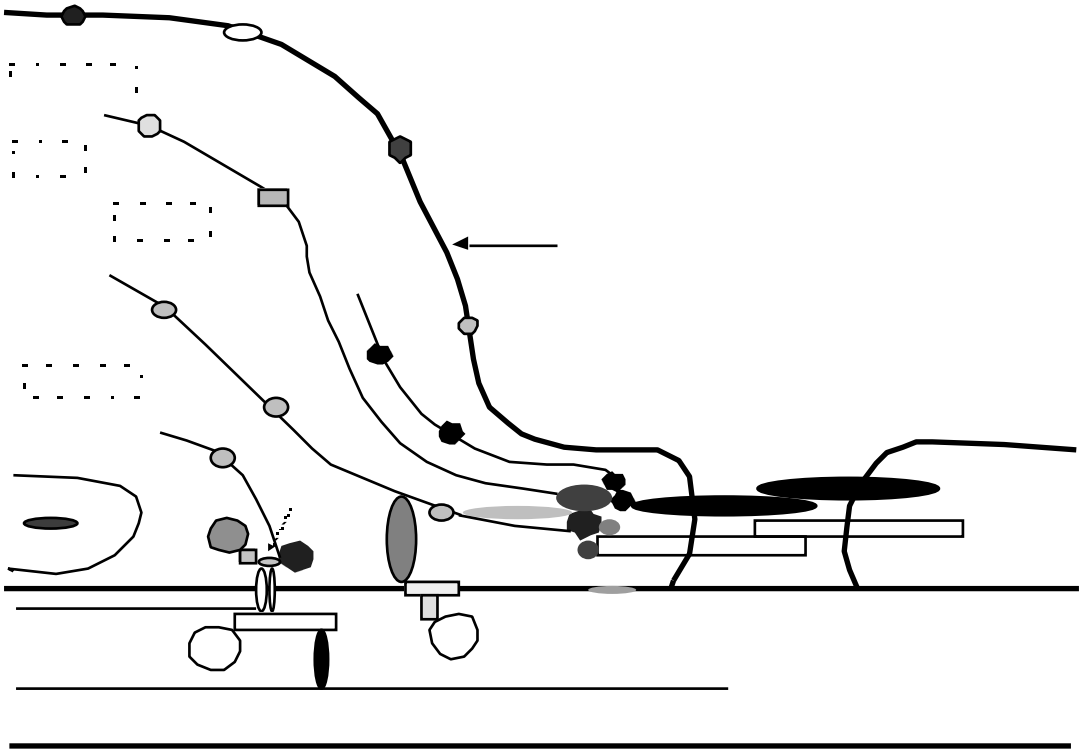

Figure 3. Molecular architecture of the slit diaphragm and podocyte. Identified molecules, such as nephrin and P-cadherin, can interact with each other by forming homodimers at the slit diaphragm to prevent macromolecules from passing easily. Nephrin and P-cadherin might bind to the crosslinking protein molecules and these molecules in turn function as the bridges to F-actin. Several other podocyte-specific and/or -important molecules are also marked. a3b1, α3β1 (integrin); a, α-catenin; b, β-catenin; g, γ-catenin; a-DG, α-dystroglycan; b-DG, β-dystroglycan; a-actinin-4, α-actinin-4 (with special reference to Raats *et al.* 2000a, Somlo and Mundel 2000)

3. Glomerular basement membrane

GBM is a specialized extracellular matrix, separated by glomerular epithelium layer outward and endothelium layer inward. This sheet-like structure is gradually formed during embryonic development, primarily by the interactions among epithelial, endothelial, and mesenchymal cells. Under EM, three layers of GBM are visible, *i.e.* a central lamina densa, lamina rara interna and externa (Saborio and Scheinman 1998). The thickness of GBM varies from 20 to 350 nm in the adult kidney (Smeets *et al.* 1996, Morita *et al.* 1989). The GBM has an average thickness of approximately 300 nm (Smeets *et al.* 1996). The main components of GBM are the collagens (type IV, V and VI), the glycoproteins (laminin, fibronectin, entactin/nidogen), and the proteoglycans (agrin, perlecan, bamacan, collagen XVIII) (Paulsson 1992). The importance of GBM has been underlined by hereditary diseases and the respective knockout mice models (Smeets *et al.* 1996, Saborio and Scheinman 1998).

Type IV collagen

The basic scaffold component of GBM is type IV collagen, to which a series of molecules are attached through molecule-molecule interactions and cross-links (Tisher and Madsen 1991, Zou 1997). So far, six different collagen subunits of collagen IV have been identified, termed α1-α6, which are encoded by genetically distinct genes COL4 A1-A6 (Smeets *et al.* 1996, Saborio and Scheinman 1998, Groffen *et al.* 1999). α1 and α2, α3 and α4, α5 and α6 have been mapped to different chromosomes: 13, 2, and X, respectively (Smeets *et al.* 1996). All six subunits enjoy the similar domain structures: a non-collagenous N-terminal 7S domain, a central triple-helical collagenous domain, and a C-terminal noncollagenous NC1 domain (Miner 1999). The function of collagen IV has been underscored by the hereditary diseases. In Alport syndrome, there exists absent or diminished one or more of three chains (α3, α4, α5), and the recurrence after kidney transplantation is due to autoantibodies primarily to α5(IV)NC1 in most patients with X-linked Alport syndrome (Smeets *et al.* 1996, Brainwood *et al.* 1998). In Goodpasture syndrome, the alloantibodies can be found towards α3(IV)NC1 (Turner and Rees 1996, Borza *et al.* 2000). The knockout mice supply the similar phenotype as showed in patients with Alport syndrome (Cosgrove *et al.* 1996).

Laminin 11

Laminins are a large glycoprotein superfamily comprising of α, β, and γ chains. So far, 12 laminins have been reported (Miner 1999). For each of them, different isoforms were described and named α1-5, β1-4, and γ1-3. Most of laminins have been found in the kidney by immunohistochemistry, but rarely in the GBM. Laminin 11 (α5β2γ1) is found only in the GBM of the kidney, indicating its essential role (Engvall *et al.* 1990). Laminin 11 has been found to bind to agrin and associate with α-dystroglycan and integrin on the podocyte side, suggesting its potential role in the glomerular filter (Denzer *et al.* 1998, Groffen *et al.* 1999). The importance of laminin β2 chain was verified, as targeted mutation of laminin β2 chain gene mice started to demonstrate nephrotic syndrome at about seven days after birth. The pathological analysis showed that there is fusion of foot processes, resembling the minimal change glomerulonephropathy (Noakes *et al.* 1995).

Entactin/nidogen

Entactin/nidogen was first purified from the extracellular matrix, and later renal entactin/nidogen from bovine renal tubular basement membrane (Katz *et al.* 1991, Groffen *et al.* 1999). It has been identified as a glycoprotein with M_r about 150 kDa, which consists of three globular domains (G1, G2 and G3) separated by two linear rod-like segments (Miner 1999). It has been found that domain G2 is closely associated with collagen IV (Aumailley *et al.* 1989) and perlecan (Groffen *et al.* 1999). Entactin/nidogen can bind to laminin by laminin γ1 chain short arm (Mayer *et al.* 1993). Recently, the mouse and human homologues of entactin/nidogen

have been reported and termed as “entactin-2” (Kimura *et al.* 1998) and “nidogen-2” (Kohfeldt *et al.* 1998). The general role of entactin/nidogen as one component of GBM has already been showed via its interactions with other molecules, but the detailed specific functions deserve further study.

Agrin

The gene for agrin, AGRN, was originally assigned to the locus 1p36.1-1pter (Rupp *et al.* 1992), being close to the perlecan gene (1p35-1p36.1). AGRN encodes agrin protein with M_r 212 kDa (Groffen *et al.* 1998a). Agrin has two regions for binding heparan sulfate, thereby, it is considered to be a heparan-sulfate-proteoglycan (HSPG) protein. Several splicing variants have been found in a tissue-specific way (Groffen *et al.* 1999). The function of agrin has been extensively investigated. It is generally thought that agrin may play several roles in the kidney. N-terminus of agrin can bind to laminin 11, and therefore strengthen the anchorage part in the GBM and provide a link with the cytoskeleton on the podocyte side; agrin can stabilize the matrix structure and contribute to the maintenance of the charge sieving of GBM; and finally it could be involved in transmembrane signalling of the cells (Groffen *et al.* 1998b, Groffen *et al.* 1999, Raats *et al.* 2000a). In diabetic nephropathy, the agrin staining diminished, indicating its roles in the pathogenesis of diabetic glomerular nephropathy (Tamsma *et al.* 1994). Agrin is a principal HSPG component of human GBM (Groffen *et al.* 1998b).

Perlecan

For a long time, perlecan was the only known HSPG associated with the basement membrane. Its distribution is wide, including Bowman’s capsule, the mesangial matrix, and GBM in the kidney, liver, and heart (Murdoch *et al.* 1994, Pyke *et al.* 1997). However, the presence of perlecan in GBM has been questioned due to a lack of firm evidence (Pyke *et al.* 1997). Perlecan has a M_r 467 kDa and consists of five domains and its corresponding gene HSPG2 is assigned to 1p35-p36.1 (Cohen *et al.* 1993). Several roles for perlecan were suggested. Perlecan was showed to participate in mitogenesis and angiogenesis; it also mediated cell attachment and functions as a resistant factor for other cell types; by binding to other molecules perlecan co-operated with them in the maintenance of normal function of glomerular filter (Groffen *et al.* 1999, Raats *et al.* 2000b).

4. Proteinuria

Protein excretion in normal adult urine is usually below 150 mg/day (80 ± 25), and at times even as high as 300 mg/day is considered to be within normal range. Urinary proteins contain mostly albumin (40%), other constituents include plasma Igs (15%), additional plasma proteins (5%), and different kinds of tissue proteins (40%) (Dennis and Robinson 1985). The semiquantitation of protein excretion in the urine can be done by measurement of the ratio between urinary protein and creatinine concentrations (Abitbol *et al.* 1990). The accurate

quantity is performed by turbidimetric or colorimetric methods. The severity of proteinuria can also be marked just with "-", "+" (≈30 mg/dl), "++" (≈100), "+++" (≈300), "++++" (≈2000) according to the dipstick test (Bergstein 1999). In agreement with the pathogenesis of proteinuria, proteinuria is divided into five classes: glomerular proteinuria, tubular proteinuria, overflow proteinuria, secretory proteinuria, and histuria.

Of these five types, glomerular proteinuria is the commonest, both physiologically and pathologically. The former is not due to the state of disease, while the latter is thought to be the dysfunction of the glomerular filtration barrier (Maack *et al.* 1985), which can manifest itself as nephrotic syndrome-like proteinuria, *i.e.*, >3.5 g/day/1.73m^2. The dominant protein in glomerular proteinuria is albumin. It can be selective or non-selective (Dennis and Robinson 1985). Glomerular proteinuria is an early sign of renal disorders, and it may in turn promote the progression of kidney diseases. Normally, the filtered load of distinct proteins are mostly reabsorbed. The dysfunction of tubular reabsorption causes tubular proteinuria, whose mechanism is not clear yet. The total protein excretion rarely surpasses 2 g/day if just tubular dysfunction exists. The main components of tubular proteinuria are β2-microglobulin and globulins. Today, β2-microglobulin of plasma and urine can be used as one of the parameters to estimate the tubular function (Maack *et al.* 1985). If the levels of any plasma proteins, such as small-size protein, are increased to some extent, they can be filtered in excess of the reabsorption capacity of normal tubules and then be present in the urine. This is called overflow proteinuria. Bence-Jones proteinuria is such an example. Histuria and secretory proteinuria are referred to as the proteins originating from surrounding tissues or other organs which can be present in the urine via excretion and secretion. Generally, the amount is small in histuria (Dennis and Robinson 1985).

5. Nephrotic syndrome

Nephrotic syndrome is a series of diseases manifested generally as heavy proteinuria (>3.5 g/day/1.73m^2), hypoalbuminemia, with or without edema and hyperlipidemia. It can be categorized as primary and secondary nephrotic syndrome, the former is due to primary glomerular diseases, while the latter is associated with specific etiologic events or a complication of other diseases. Congenital nephrotic syndrome is generally thought to be a group of diseases belonging to secondary nephrotic syndrome.

5.1. Congenital nephrotic syndrome

Congenital nephrotic syndrome is often refered to as a type of nephrotic syndrome occuring during the first three months of life. Besides the acquired type, there exist four classes of congenital nephrotic syndrome, *i.e.*, CNF, diffuse mesangial sclerosis, idiopathic nephrotic syndrome (minimal change and FSGS), and syndromic congenital nephrotic syndrome (Drash

syndrome, Nail-Patella syndrome) (Smeets *et al.* 1996, Holmberg *et al.* 1996). The commonest type is CNF, mainly found in Finland.

5.1.1. Congenital nephrotic syndrome of the Finnish type

In 1956, Hallman *et al.* (1956) first reported a specific type of nephrotic syndrome discovered in infants. Later, it was found that this disease is mainly restricted to Finland, and was thus named "congenital nephrotic syndrome of the Finnish type" or "CNF". CNF is the first identified disease which belongs to the Finnish disease heritage. Since then on, CNF cases have also been found in many other countries, but mainly centralized in Europe and USA (Mahan *et al.* 1984, Bucciarelli *et al.* 1989, Savage *et al.* 1999, Aya *et al.* 2000). In the Chinese population, several cases have been reported (Xu 1988, Lin *et al.* 1997).

Clinical characteristics

CNF is inherited as an autosomal recessive disease (Norio 1966, Huttunen *et al.* 1976). Its incidence in Finland is about 1.2 per 10,000 live births (Huttunen *et al.* 1976). The affected children are usually born prematurely, ranging from 35^{th} to 38^{th} gestation weeks, with proteinuria even starting *in utero* (Huttunen *et al.* 1975, Lenkkeri 1998). The placenta is large and weights more than one quarter of the birth weight. Proteinuria is extremely severe, accompanied by apparent hypoalbuminemia and very high hyperlipidemia. Over half of the infants have edema in the first week (Antikainen *et al.* 1992, Holmberg *et al.* 1995, Holmberg *et al.* 1996). CNF infants, whether pre- or post-transplantation, quite often suffer from different kinds of infection, the former is thought to be due to the urinary loss of Igs and complement factors B and D, while the latter is due to the use of immunosuppressive agents as in other transplantation cases. In addition, thromboembolism and seizure are common (Huttunen *et al.* 1976, Mahan *et al.* 1984, Laine *et al.* 1993). Additional features are mainly hypothyroidism, umbilical herniae, bony deformities, and developmental delay (Hallman *et al.* 1976). During the first six months, glomerular filtration rate is within normal range, but later, a fall is not avoided (Holmberg *et al.* 1996).

Pathological findings

In CNF infants, the size of the kidney is about 2-3 times larger than normal and the glomerulus volume is almost double the normal size. The total number of glomeruli is increased and also the areas of normal size glomeruli are tightly compacted in clusters, which suggest a failure in the coordinated mesenchymo-epithelial interaction during nephrogenesis (Tryggvason 1978, Huttunen *et al.* 1980, Haltia *et al.* 1998). The ratio of the mature to immature glomeruli increases, while the percentage of the podocyte cells decreases (Autio-Harmainen and Rapola 1981). In the later stage of development, glomerulosclerosis and mesangial hypercellularity have been noticed. Tubular atrophy and irregular dilations of proximal convoluted tubules have also been observed, therefore, CNF was called "microcystic disease" (Huttunen *et al.* 1980, Rapola

1981). On EM, the major characteristic is the obvious fusion and flattening of foot processes of the podocytes (Autio-Harmainen 1981a&b). Immunofluorescence has not revealed any deposits of Ig and complements (Lenkkeri 1998).

Pathogenesis

The pathogenesis of CNF has been systematically investigated. Haltia *et al.* (1998) found abnormal renal differentiation, contributing to excessive and poorly organized formation of the glomeruli. Several important genes (WT1, PAX2, EGR1, IGF1 and 2, VEGF) were not found to be abnormal in the podocyte cells (Haltia *et al.* 1996, Haltia *et al.* 1997). The thickness of GBM has been studied by Autio-Harmainen and Rapola (1983) and Ljungberg *et al.* (1993). Interestingly, their results were contradictory. Matrix components such as laminin, fibronectin, entactin, proteoglycan have also been studied extensively (Ljungberg *et al.* 1993, 1996a&b). The work on charge- or size-related GBM does not lead to a clear conclusion. Vernier *et al.* (1983) found a decrease in glomerular anionic charge of the GBM. However, this lacks the support from van den Heuvel *et al.* (1992) and Ljungberg *et al.* (1995).

Prenatal and postnatal diagnosis

Traditionally, the prenatal diagnosis of CNF can be crudely made on the base of high α-fetoprotein in maternal serum and/or in amniotic fluid by the second trimester of pregnancy (Holmberg *et al.* 1996). However, high α-fetoprotein is not specific for CNF, it can also be found in other disorders such as neural tube defect, Turner's syndrome, abortion (Ryynänen *et al.* 1983, Lenkkeri 1998). According to one systematic study (Holmberg *et al.* 1996), postnatal diagnosis is made based upon the following indexes: (1) positive family history; (2) high α-fetoprotein concentration; (3) megaplacenta (>25% of birth weight); (4) onset of severe proteinuria *in utero* (serum albumin <10 g/l at presentation and proteinuria >20 g/l when serum albumin is corrected to >15 g/l); (5) elimination of other types of congenital nephrotic syndrome; (6) normal glomerular filtration rate during the first 6 months. With the discovery of nephrin, prenatal diagnosis based on the haplotype analysis is possible and it can be made as early as 12^{th} to 13^{th} gestational weeks, moreover, this method is specific (Kestilä *et al.* 1994a, Lenkkeri 1998, Kestilä *et al.* 1998). In this way 95% accuracy can be obtained (Lenkkeri 1998). Mutation analysis by PCR and dual-colour oligonucleotide ligation assay can be used to screen the suspected infants (Romppanen and Mononen 2000).

Treatment

The treatment of CNF is relatively clear. Kidney transplantation is the only curative therapy, in combination with the supportive medication. Before kidney transplantation, bilateral nephrectomy is performed in order to end the proteinuria, and continuous cycling peritoneal dialysis is taken for several months to correct the protein and lipid status and to get the children into an better nutritional state. Some centres perform unilateral nephrectomy to make the

substitution easier, but some investigators (Coulthard 1989) thought that this could accelerate the development of uremia.

5.2. Primary nephrotic syndrome

Primary or idiopathic nephrotic syndrome is a group of diseases with the typical characteristics of nephrotic syndrome, but their causes are usually unknown. According to histologic lesions, primary nephrotic syndrome can be categorized as several classes, *i.e*., MCGN, non-IgA mesangial proliferative glomerulonephritis (MsPGN), IgA nephropathy, FSGS, MGN, membranoproliferative glomerulonephritis (MPGN), endocapillary proliferative glomerulonephritis, and unclassified lesions.

5.2.1. Minimal change glomerulonephropathy

Minimal change glomerulonephropathy is mainly characterized by the effacement and retraction of the podocytes pathologically, and massive proteinuria clinically. MCGN used to be called “lipoid nephrosis” by Munk, because lipid droplets were found in the proximal tubular cells. Several other terms, such as “minimal change lesion”, “minimal change disease”, and “minimal change nephropathy”, are also widely used nowadays to stress the relative paucity of glomerular changes under light microscopy. MCGN usually occurs in young children with an incidence peak from 2 to 6 years. MCGN accounts for about 80% and 30% cases of the primary nephrotic syndrome in children and adults respectively. The ratio of male/female is 2:1 (Glassock *et al.* 1991a). MCGN was reported to be more common in Asian population than others (Sharples *et al.* 1985).

Laboratory findings reveal that heavy proteinuria, often exceeding 40 mg/hr/m^2, exists, accompanied by hematuria found in 15-20% of the cases (Glassock *et al.* 1991a). Serum albumin is often decreased, even to less than 10 g/l. Total cholesterol, triglycerides, very low density lipoprotein and low density lipoprotein levels are generally increased (Glassock *et al.* 1991a, Wang 1997). Edema is found quite often and could be the initial presenting sign in the clinic. In adults, the clinical features are different from those seen in children in several ways: (1) insidious onset; (2) hypertension and hematuria; (3) high proportion of renal damage and slow recovery; (4) slow response or even resistance to steroid treatment (Coleman and Ruef 1992, Wang 1997).

Pathological characteristics are not so many as for other types of primary nephrotic syndrome. Light microscopy indicates that the glomeruli are largely normal, with a mild increase of cellularity of the mesangium and enlargement of epithelial cells; the proximal tubules may contain fine lipid droplets that are doubly refractile. Interestingly, these changes can also exist in patients with early membranous glomerulonephritis (Glassock *et al.* 1991a). At times, they are very difficult to be distinguished. The findings under IF microscopy are a lack of deposits of Ig

and complement. Occasionally, IgM deposit is seen in the mesangium (Wang 1997). EM shows effacement of the epithelial podocyte cells with vacuolization and microvillous transformation (Wang 1997). The obliteration of the slit pore membrane complex is also noted in most glomeruli and glomerular capillaries (Glassock *et al.* 1991a). These clinical and pathological findings have been mimiced in a rat PAN model, which is widely utilized to explore the pathogenesis of MCGN. The loss of negative charge in the capillaries is responsible for the proteinuria though the reason is unknown (Guasch *et al.* 1991). Several factors have been considered to explain the dysfunction of the glomerular permselectivity: (1) alterations of charge of GBM-related molecules (Kanwar and Farquhar 1979, Garin and Corontzes 1992, Holthöfer *et al.* 1996); (2) glomerular permeability factors (Ambrus and Sridhar 1997, Couser 1998); and (3) lymphokines (Wang 1997). In a steroid-resistant model of Mpv17 gene-inactivated mouse, scavengers for reactive oxygen species (ROS) significantly reduced proteinuria, suggesting its relationship with the overproduction of oxygen radicals (Binder *et al.* 1999, Wang *et al.* 2001). In addition, many clues indicate that T cell disorder is involved in MCGN and the formation of membrane attack complex (Schnaper 1989, Nangaku *et al.* 1999).

The treatment of MCGN is well established and the prognosis generally good if the patients have the following features: (1) minimal glomerular changes by light microscopy; (2) diffuse epithelial cell lesion only by EM; (3) absence or minimal deposition of Igs by IF; and (4) a complete remission following a course of steroid therapy (Glassock *et al.* 1991a). So far, corticosteroid is the first choice and the major therapy. On the first attack, prednisone or prednisolone is suggested at 60 mg/m^2/day (even up to 80 mg/m^2/day) for four to six weeks, followed by 40 mg/m^2/day of prednisone every other day for another four to six weeks (Bargman 1999). Most patients will clear their proteinuria by two weeks (ISKDC 1981). Adults may in contrast respond more slowly than children, so more than 8 weeks may be needed to ascertain the steroid responsiveness (Korbet *et al.* 1988). The biggest challenge during therapy is relapse. For initial relapse treatment, prednisone should be dosage- and time-enough until the proteinuria disappears for at least three days, and then an alternate day regimen of 40 mg/m^2/day should be used for another month (Bargman 1999). Sometimes, cyclophosphamide and chlorambucil should be considered, and usually a 8- or 12-week course of the former (Bargman 1999). If there exists steroid-dependent or resistant, besides cyclophosphamide administration, cyclosporine is also adopted (Bargman 1999).

5.2.2. Mesangial proliferative glomerulonephritis

According to the components of deposits in the mesangium, MsPGN could be categorised as non-IgA glomerulonephritis and IgA nephropathy entities (Glassock *et al.* 1991a). The former accounts for the largest fraction (25-31%) of primary nephrotic syndrome

cases in China, comparing with around 10% of all the cases in Europe and North America (Chen *et al.* 1989, Glassock *et al.* 1991a).

5.2.2.1. Non-IgA glomerulonephritis

This type of nephrotic syndrome is relatively unevenly distributed and uncommon around the world. One major feature of non-IgA glomerulonephritis is its insidious onset (Bhasin *et al.* 1978, Glassock *et al.* 1991a). Hematuria is found in the majority of the cases, and mild hypertension is present in only about 30% of cases (Glassock *et al.* 1991a). Proteinuria is often non-selective (Poucell *et al.* 1985). Hypertension and decrement of kidney function are usually discovered in severe non-IgA glomerulonephritis patients (Chen *et al.* 1989).

Light microscopy findings show that an increase in cellularity of the mesangium and mesangial matrix is an early dominant characteristic of non-IgA glomerulonephritis (Glassock *et al.* 1991a). Under EM, finely granular or homogeneous electron-dense deposits are found in the mesangium in about half of the biopsies, the GBM is generally normal (Glassock *et al.* 1991a). IgM and C3 sedimentation are frequently found in the mesangium. Therefore, Cohen *et al.* (1978) named it as "IgM mesangial nephropathy". Aside from IgM deposits, IgG deposits can be found in 57-60% of the cases in China (Chen *et al.* 1989).

The pathogenesis of non-IgA glomerulonephritis is not fully known. Based upon the observation on IgM and C3 deposits as well as circulating immune complex, non-IgA glomerulonephritis appears to be an immune complex disease (Glassock *et al.* 1991a). An anti-Thy-1.1 rat model shows similar changes in the mesangium of the glomeruli manifesting as massive proliferation of mesangial cells at the late stage, though characteristics such as mesangiolysis at the early stage is not completely the same as the findings in human (Bagchus *et al.* 1986, Jefferson and Johnson 1999). A variety of factors, such as interleukin-1, TGF-β, and platelet-derived growth factor, might participate in the mediation of mesangial cell proliferation (Tesch *et al.* 1997, Jefferson and Johnson 1999). More recently, signalling pathways in cultured mesangial cells and in rat model have been studied (Nakashima *et al.* 1999, Bokemeyer *et al.* 2000).

For the treatment of non-IgA glomerulonephritis, a similar strategy with MCGN can be used, particularly if extensitive IgM or C3 deposits are absent and the proliferation is mild (Wang 1997). If the patients have superimposed FSGS on initial or follow-up biopsies, a poor prognosis is possible (Glassock *et al.* 1991a).

5.2.2.2. IgA nephropathy

Another common type of MsPGN is IgA nephropathy, reported originally by Berger and Hinglais (1968), also named "Berger's disease". IgA nephropathy is quite common in Asian-Pacific areas (30-40%) and Europe (20%), and nowadays is even considered to be the

commonest variety of primary glomerular disease worldwide (Clarkson *et al.* 1984 and 1988, D'Amico 1987, Glassock *et al.* 1991a, Nolin and Courteau 1999). Male predominates in IgA nephropathy, the ratio of male/female ranging from 2:1 to 6:1 (Clarkson *et al.* 1984).

As its name implies, IgA nephropathy is defined as prominent and diffuse granular deposits of IgA in the glomerular mesangium of all biopsies on IF (Nolin and Courteau 1999). In many cases, IgG can also be concomitantly found in glomerular mesangium besides IgA, so sometimes this mixed entity can be called "IgG/IgA nephropathy" (Glassock *et al.* 1991a). Under light microscopy, IgA nephropathy has variable changes in glomeruli, but proliferation of mesangial cells and matrix is the commonest feature (Sakai 1991). With regard to the severity of the disease, according to WHO standards in 1982, IgA nephropathy is classified as five types (Schena 1992). In almost all biopsies, finely granular to homogeneous electron-dense deposits are found in the mesangium, accompanied by hypercellularity and proliferation of mesangial matrix. These are the typical findings of IgA nephropathy (Glassock *et al.* 1991a).

The pathogenesis of IgA nephropathy is unknown and perhaps related with many factors. Inflammation strongly contributes to it. Several lines of evidence indicate that IgA nephropathy is some kind of immune complex glomerulonephritis. However, the origin of antigens responsible for the development of IgA nephropathy is not clear (Clarkson *et al.* 1984). Several candidates have been suggested, such as virus-like antigens in the upper respiratory tract, soybean protein (Sakai 1991). Recently, interleukin-6 was found to have a potent capacity to make mesangial cells proliferate not only *in vitro*, but also *in vivo* (Sakai 1991). The effects of ROS on glomeruli in IgA nephropathy have been reported (Johnson *et al.* 1986). In addition, the dysfunction of hemodynamics of the kidney could also contribute to the pathogenesis of IgA nephropathy due to the presence of angiotensin II receptor in the glomeruli (Woodroffe *et al.* 1987). IgA nephropathy was also found in the siblings, therefore genetic factors can not be completely ruled out (Schena 1992).

Many ways have been suggested to treat IgA nephropathy (Locatelli *et al.* 1999, Nolin and Courteau 1999, Julian 2000). Prednisone is always the first choice. Cyclophosphamide, dipyridamole, warfarin, and cyclosporine A could also be considered in some situations. Fish oil is a beneficial addition. At times azathioprine and prednisone can be used in combination. Angiotensin II inhibitor can also be used. Tonsillectomy might be beneficial in IgA nephropathy patients with recurrent tonsillitis. In one report, it was said that 20-30% of patients developed progressive renal insufficiency 20 years or more after initial discovery of disease. Clinical features, which indicate a poor prognosis, include male sex, late age onset, decreased glomerular filtration rate at discovery, persistent nephrotic range proteinuria, and moderate hypertension (Glassock *et al.* 1991a).

5.2.3. Focal segmental glomerulosclerosis

Rich (1957) first described the pathologic findings of FSGS in an autopsy study in 1957. However, some investigators still think FSGS is a non-specific lesion superimposed on other lesions, such as MCGN or MsPGN (Kashgarian *et al.* 1974). FSGS occurs both in children and adults, accounting for 7-15% and 15-20% cases respectively of the primary nephrotic syndrome. The proportion of cases in male is slightly larger compared to female. Hematuria, whether microscopic or macroscopic, is common. The non-selective proteinuria could be found in a small proportion of FSGS patients (White and Glasgow 1971).

Pathological findings on light microscopy show focal and segmental sclerosis lesions affecting a variable minority of the glomeruli, usually those in deeper, juxtamedullary cortex due to the higher perfusion pressure (Glassock *et al.* 1991a). Foamy lipid-laden histiocytes are distributed sporadically in the glomerular mesangium or glomeruli (Glassock *et al.* 1991a, Wang *et al.* 1997). EM examination present, in most cases, the characteristics of the effacement of podocytes and the detachment of foot processes from the GBM, especially if heavy proteinuria is observed (Glassock *et al.* 1991a). In the sclerosis region, there exists IgM and C3 deposition, nonetheless, IgM and C3 can also be found in the normal glomeruli (Jacquot *et al.* 1981, Glassock *et al.* 1991a).

The pathogenesis of this disorder is obscure. Albumin administration could cause similar glomerular changes, suggesting a role of macromolecule absorption by mesangial cells (Marks and Drummond 1970). Subnephrectomy rats could develop segmental and focal sclerosis, indicating the crucial role of kidney hemodynamics (Brenner 1983). The relationship between lipid and glomerular sclerosis has recently attracted more attention. This hypothesis is based upon the following evidence: (1) high lipid diet can result in sclerosis of glomeruli in the rat (Grone *et al.* 1989); (2) the Zucker rat is able to develop glomerulosclerosis spontaneously (Kasiske *et al.* 1985); (3) treatment of hyperlipidemia can reduce glomerular injury in obese Zucker rats (Kasiske *et al.* 1988). In addition, infiltration of macrophages in the glomeruli (Magil and Cohen 1989), dysfunction of coagulation (Purkerson *et al.* 1985), and podocyte cells involvement (Nagata and Kriz 1992, Schwartz *et al.* 1999) are all proposed. Finally, genetic factor has been noticed.

Treatment with prednisone should be considered in all patients and continue for half a year before considering the resistance to therapy (Burgess 1999, Schena 1999). However, in one study most of the patients didn't respond well (Wang 1997). Cyclosporine A can be used for reducing proteinuria. Relapse after decreasing or stopping cyclosporine A is very common (Burgess 1999). Cytotoxic therapy (cyclophosphamide, azathioprine and chlorambucil) for adults is an alternative choice if the above treatment is ineffective. Finally, plasmapheresis or protein adsorption may be used for the patients with recurrent FSGS (Burgess 1999).

5.2.4. Membranous glomerulonephropathy

Membranous glomerulonephropathy refers to a specific entity of primary nephrotic syndrome associated with diffuse deposits of immune complexes under the epithelium and thickening of the GBM. It is also termed as "epimembranous, perimembranous, or extramembranous glomerulonephritis". MGN is a leading cause of primary nephrotic syndrome in adults (Muirhead 1999, Shankland 2000), though it can be found at any age. The onset of MGN is usually insidious and presented as gradual edema of the legs (Wang 1997). Most patients have heavy proteinuria (>3.5 g/day) with or without hematuria, accompanied by hypoproteinemia; hyperlipedimia (triglycerdemia, lipoproteinemia) is less common (Wasserstein 1997).

The typical feature of MGN is charaterized by diffuse and uniform thickening of the GBM, usually without any significant proliferation of endothelial, mesangial, or epithelial cells (Ambrus and Sridhar 1997). The findings on EM reveal subepithelial and intramembranous electron-dense deposits (Ambrus and Sridhar 1997). According to the changes identified by light microscopy and EM, some researchers have found a series of changes which correspond to the clinical phases. These have been divided into four stages (I-IV), which are associated closely with the prognosis of the disease (Glassock *et al.* 1991a). Immunofluorescence reveals, in almost all the cases, that IgG and C3 are present in a uniform granular distribution of all the capillary loops (Glassock *et al.* 1991a).

The pathogenesis of MGN is not known clearly. Rat model of Heymann nephritis has been widely used for the exploration of the mechanism, owing to its similarity with the features of human MGN (Heymann *et al.* 1959, Salant *et al.* 1980, Miettinen *et al.* 1980). Several possibilities have been proposed: (1) circulating immune complex found in patients and animal models (Couser 1981, Gallo *et al.* 1981). However, no evidence has yet proven that the circulating immune complex is the same as that deposited in glomeruli (Ooi *et al.* 1977); (2) formation of immune complex *in situ.* gp330 is suspected to interact with its antibody and exists in clathrin-coated pits under the epithelium (Miettinen *et al.* 1980, Makker 1993, Orlando *et al.* 1995); (3) formation of membrane attack complex (Couser *et al.* 1992). Several signal pathways have been reported to be involved when the holes are created (Cybulsky *et al.* 2000) ; (4) T cell disorder (Pruchno *et al.* 1991); (5) extracellular matrix changes. The thickening of the GBM in MGN is possibly due to the accumulation of extracellular matrix proteins (Shankland 2000). TGF-β and their receptors are potential mediators of matrix accumulation in Heymann nephropathy (Shankland *et al.* 1996).

Effective management protocols for MGN have been recommended by Muirhead (1999) and Geddes and Cattran (2000). The use of corticosteroids is controversial, based upon three independent clinical trials (CSAINS 1979, Cameron *et al.* 1990, Cattran *et al.* 1989). Spontaneous remissions can occur in about 25% of patients (Glassock *et al.* 1991a). Hence, the

use of corticosteroids should be considered carefully when in use. In some situations, corticosteroids and alkylating agents can be combined for prolonging remission of MGN (Muirhead 1999). Cyclosporine therapy shows promising effects on MGN patients, particularly for those who are at high risk for progressive renal failure (Muirhead 1999).

5.2.5. Membranoproliferative glomerulonephritis

Membranoproliferative glomerulonephritis is also known as: “mesangiocapillary glomerulonephritis”, “hypocomplementemic persistent glomerulonephritis”, “lobular glomerulonephritis”, or “chronic mesangioproliferative glomerulonephritis” (Glassock *et al.* 1991a). MPGN is mainly found in children and adolescents rather than in the elderly (Cameron *et al.* 1983), who constitutes the smallest proportion and accounts for only 10-20% of cases of the primary nephrotic syndrome (Glassock *et al.* 1991a). The clinical features are rather varied. Many patients present heavy proteinuria initially, accompanied by gross or microscopic hematuria (Ambrus and Sridhar 1997). A small proportion of patients can be found to have, usually mild hypertension (Wang 1997). Kidney impairment is uncommon in MPGN patients (Bosch *et al.* 1986).

Based on the ultrastructural and immunofluorescence patterns, idiopathic MPGN can be further categorized as three subtypes: I, II, and III (Glassock *et al.* 1991a, Wang 1997). In type I, subendothelial and mesangial electron-dense deposits are noted. Immunocomplexes can be IgG, C3, C1q or C4. This type accounts for 65-75% of all MPGN patients. In type II, electron-dense deposits can be found in the mesangium and within the GBM. C3 is predominant in immune complex deposits, IgG is not usually found. This type accounts for about 20-30% of all cases. Sometimes, type II is also named “dense deposit disease”. On light microscopy, type I and type II are very difficult to distinguish. Type III is very rare. The electron dense deposits are mainly found under endothelium, epithelium, and in the mesangium. C3 is predominant in immune complexes, accompanied by IgG. The pathogenesis has been studied, but no convincing theory was established. Lack of successful animal models further restrict delineation of the mechanism. It is generally thought that MPGN is a type of immune complex disease (Ambrus and Sridhar 1997) and also genetically related (Bogdanovic 2000). In summary, the pathogenesis of MPGN is not clear and maybe herterogeneous due to the diversity characteristics in pathology.

Six to twelve months steroid therapy has been proven effective for children but not for adults, in order for reduction of proteinuria. Cyclosphosphamide, aspirin, dipyrimadole, and warfarin sodium have been used for mild MPGN (Levin 1999).

5.3. Secondary nephrotic syndrome and systemic diseases

This group of diseases are caused by various etiologies already known (**Table 1**).

Table 1 The commonest causes of secondary nephrotic syndrome (adapted from Glassock *et al.* 1991b)

Medications	
	Organic, inorganic, elemental mercury
	Organic gold
	Penicillamine
	Heroin
	Probenecid
	Captopril
	Nonsteroidal anti-inflammatory drugs
Allergens, venoms, immunizations	
	Bee sting
	Pollens
Infections	
	Bacteria Poststreptococcal glomerulonephritis, infective endocarditis, shunt nephritis
	Virus Hepatitis B
	Protozoal Malaria
	Helminthic Schistosomiasis
Neoplastic	
	Solid tumors Lung, colon, stomach, breast
	Leukemia and lymphoma Hodgkin's disease
Multisystem disease	
	Systemic lupus erythematosus
	Schönlein-Henoch purpura
	Amyloidosis
Heredofamilial and metabolic disease	
	Diabetes mellitus
Miscellaneous	
	Chronic renal allograft rejection

Table 2 Recurrent and *de novo* kidney diseases (adapted from Ramos *et al.* 1991, Markowitz *et al.* 1998, Denton and Singh 2000)

Recurrent	*De Novo*
IgA nephropathy	
FSGS	
MGN	MGN
MPGN I and II	MPGN
	MCGN
Systemic lupus erythematosus	
Anti-GBM disease	Anti-GBM disease in Alport syndrome
CNF	
Schönlein-Henoch syndrome	
Hereditary nephritis	
Hemolytic uremic syndrome	Hemolytic uremic syndrome
Wegener's granulomatosis	
Diabetes mellitus	
Amyloidosis	
Oxalosis	
Fabry's disease	
Sickle cell nephropathy	
Progressive systemic sclerosis	
	Transplant glomerulopathy

6. Recurrent and *de novo* glomerular diseases in kidney transplantation

Heavy glomerular proteinuria is not only found in primary nephrotic syndrome, but also after kidney transplantation. Proteinuria after transplantation generally contributes to three types of causes: recurrent or *de novo* glomerulonephritis, and chronic rejection (Ramos *et al.* 1991, Laine *et al.* 1993, Hariharan *et al.* 1999, Denton and Singh 2000) (**Table 2**).

Recurrence of glomerulopathy was reported firstly by Hume *et al.* (1955). It can be observed both in primary kidney diseases and in systemic disorders. Of the primary renal diseases, IgA nephropathy is the commonest form, the proportion of recurrence varying from 26% to 50% and even as high as 60% (Frohnert *et al.* 1997, Bumgardeven *et al.* 1998, Floege *et al.* 1998). Recurrence of IgA nephropathy often manifests itself as proteinuria exceeding 0.5 g/day and continuous microhematuria. The prognosis is good for IgA transplantation patients; only 10-15% recurrence can cause graft loss (Denton and Singh 2000). Therefore, measures have to be taken to prevent the risk factors from progression to end stage renal disease. Several factors, such as heavy proteinuria, mesangial proliferation, glomerulosclerosis, and hypertension, have to attract more attention in treating this kind of patients, as these might contribute to the progression of renal disease (Kimata *et al.* 1996).

Another type of high recurrence rate in primary nephrotic syndrome is FSGS. Recurrence occurs very soon after renal transplantation (Noël 1999). Generally, the recurrence takes place within the first month and rarely later than one year after transplantation (Denton and Singh 2000). It was reported that the recurrence rate is around 30-50% (Hariharan *et al.* 1999). Several hypotheses have been suggested to explain the pathogenesis. It was suspected that a series of circulating factors exist, based upon the following evidence: (1) the short median time to recurrence (Schachter and Strom 1999); (2) effectiveness of protein A adsorption (Dantal *et al.* 1994) and plasmapheresis (Savin *et al.* 1996) therapy in reducing the proteinuria of recurrent FSGS patients. Analysis of biopsy specimens from recurrent FSGS patients has showed that gene expression of NF-κB subunit p65 is significantly elevated, and so is the intragraft angiotensinogen expression (Schachter and Strom 1999).

Membranous glomerulonephritis can appear as either recurrent or *de novo* glomerulonephritis. The prevalance of the former is around 26% (Couchoud *et al.* 1995), while the latter much lower, 1-2% (Denton and Singh 2000). Pathologically, they are difficult to distinguish from each other, thereby, the diagnosis heavily depends upon the clear pre-diagnosis. In addition, *de novo* MGN was reported to be associated with hepatitis B or C infection (Levy and Charpentier 1983). The outcome of recurrence in MGN is poor as the graft loss rate is 38% and 52% at 5 and 10 years (Cosyns *et al.* 1998).

In type I MPGN, the recurrent rate is about 25%, while in type II it is much higher, around 88% (Cameron 1982). It should be pointed out that sometimes recurrence in type I is difficult to diagnose compared with transplant rejection glomerulopathy on light microscopy.

Deposition of crescents and C3 are found in both states (Ramos 1991). Plasmapheresis can be used for treatment of these two kinds of patients, though not much experience has so far accumulated (Denton and Singh 2000).

Anti-GBM disease occurs only when anti-GBM titers exist before transplantation (Ramos *et al.* 1991). *De novo* anti-GBM disease in Alport syndrome may arise (Brainwood *et al.* 1998). The primary target of antibodies in X-linked Alport syndrome is the α5 C-terminus, which is planted when the transplant is installed (Brainwood *et al.* 1998). However, only a small number of transplant patients due to Alport syndrome develop detectable antibodies (Denton and Singh 2000).

In CNF patients, proteinuria after transplantation has been noticed (Lane *et al.* 1991, Laine *et al.* 1993, Holmberg *et al.* 1995). Laine *et al.* (1993) reported 29 transplantations of 28 CNF patients. Between 1 and 33 months, seven grafts (24%) of six patients developed heavy proteinuria similar to nephrotic syndrome. At the onset of proteinuria, there was evidence indicating six of seven episodes preceded by cytomegalovirus or Epstein-Barr virus infection and the remaining had a sinus infection. With steroid or cyclophosphamide treatment, only two patients had remission, while the other four lost their grafts.

7. Protein kinase C

Protein kinase C was discovered by Takai *et al.* (1977). So far, at least 11 members have been identified and all of them compose the PKC superfamily. PKC can phosphorylate a multitude of cellular substrates and itself can be phosphorylated (Parekh *et al.* 2000). It has been found that the PKC superfamily is involved in a bewildering array of biological processes (Nishizuka 1986 and 1992, Mellor and Parker 1998).

7.1. Isotypes and characteristics of protein kinase C

In agreement with structural and biochemical characteristics, the PKC superfamily can be grouped into three large groups: classic PKC (cPKC), novel PKC (nPKC), and atypical PKC. The cPKC consists of four isoforms (α, β1, β2, γ), which can be activated by Ca^{2+} and/or diacylglycerol and phorbol esters. The nPKC is composed of four isotypes (δ, ε, η, θ), which can be activated by diacylglycerol and phorbol esters but are Ca^{2+}-independent. Finally the atypical PKC (ζ, ι, λ) is unresponsive to Ca^{2+} and diacylglycerol/phorbol esters. They all share in common regulatory and catalytic regions, the former being an N-terminal membrane targeting moiety, while the latter being a C-terminal kinase domain (Newton and Johnson 1998). The regulatory area can be further divided into C1 and C2 domains. All the PKC isotypes contain one or more C1 domains of two-repeated zinc finger motifs (C1a and C1b). The C1 domain can play its role by binding to diacylglycerol and phorbol ester, and the C2 domain is present in cPKC isoforms, which binds Ca^{2+}, acidic lipids, phospholipases, and GTPase-activating proteins

(Hurley and Grobler 1997, Newton and Johnson 1998). In the catalytic area, C3 domain comprises the ATP binding lobe and C4 substrate binding lobe. Within regulatory and catalytic domains of PKC scatteredly lie variable regions (V1 to V5). In addition, PKC-μ/PKD (human/mouse) were reported by two independent groups several years ago (Johannes *et al.* 1994, Valverde *et al.* 1994), and so were the PKC-related kinases (Mellor and Parker 1998, Ron and Kazanietz 1999).

7.2. Kidney distribution of protein kinase C

In the kidney glomeruli, C-α can be found in normal rat glomerular epithelium (Pfaff *et al.* 1999), endothelium, and mesangial cells (Babazono *et al.* 1998). Compared to C-α, C-β1 and -β2 are not expressed in the kidney (Wetsel *et al.* 1992). However, Pfaff *et al.* (1999) and Babazono *et al.* (1998) reported their different findings. C-β1 could be detected in the mesangial cells of kidney glomeruli, proximal tubules, and medulla; C-β2 just in interstitial cells of kidney cortex and collecting duct (Pfaff *et al.* 1999). There is some controversy about whether these two subtypes exist in the cultured mesangial cells. Ganz *et al.* (1996) identified the expression of C-β1 but not of C-β2 in cultured mesangial cells. C-δ is present in the kidney (Wetsel *et al.* 1992). Immuno-EM showed C-δ present in all three glomerular cell types, *i.e.*, epithelial, endothelial, and mesangial cells (Babazono *et al.* 1998). C-ε is also detectable in the kidney (Babazono *et al.* 1998). C-ζ was found by Western blotting in the kidney of the rat (Wetsel *et al.* 1992). However, in rabbit, C-ζ was not found in the glomerulus, but in proximal tubule, thick limb, and collecting duct (Hao *et al.* 1997). In developmental study, the data in fetal mice indicated that C-α, C-β1, C-ζ, C-η, and C-θ could be detected in the kidney (Bareggi *et al.* 1995).

AIMS OF THE STUDY

The specific aims were as follows:

-To localize nephrin molecules in the glomerulus;

-To elucidate the intracellular pathways of nephrin regulation;

-To find the mechanisms for recurrence of nephrotic syndrome in CNF patients with kidney transplantation;

-To map the expression patterns of nephrin and 18C7 antigen in human renal diseases.

MATERIALS AND METHODS

The materials and methods have already been in detail described in the respective "materials and methods" parts of the original articles. The articles are referred by their Roman numbers I through IV.

Human kidney biopsies (I, III, IV)

Kidney biopsies of CNF patients were taken at nephrectomy. For normal controls, cadaver kidneys unsuitable for transplantation due to vascular anatomical reasons were used. One hundred and twenty human kidney biopsies (**Table 3**) were taken from the routine diagnostic samples at San Carlo Borromeo Hospital (Milan, Italy).

Table 3 One hundred and twenty human kidney biopsies

Diagnosis	Number
Acute post-streptococcal nephritis	2
MCGN	4
FSGS	11
MGN	20
IgA nephropathy	40
MPGN	6
MsPGN (IF negative)	2
Schönlein-Henoch syndrome	6
Systemic lupus erythematosus	10
Cryoglobulinemic glomerulonephritis	4
Renal vasculitis	2
Anti-GBM glomerulonephritis	1
Amyloidosis	3
Nephroangiosclerosis	4
Acute interstitial nephritis	2
Acute tubular necrosis	1
Alport syndrome	2

RNA isolation (I, II)

The total RNA isolation from kidney tissues and A293 cells was done according to the manufacture's instructions using TRIZOL® Reagent (Life Technologies, New York, NY, USA) and RNeasy Mini Kit (QIAGEN Inc., CA, USA).

RT reaction and semiquantitative PCR (I, II)

Before RT reaction, total RNA samples were first treated by DNase (DNase RQ1, Promega, Madison, WI, USA). The reverse transcriptase of moloney murine leukemia virus (Promega) was used for reverse transcription. PTC-200 thermal cycler (MJ Research Inc., Watertown, MA, USA) was used for cDNA amplification. The semiquantitation of nephrin was done by using serial dilutions of sample cDNA in the linear range of amplification and normalization to the amount of β-actin product.

Peptide design and polyclonal antibodies (I-IV)

The polyclonal antibodies to the intracellular (aa 1101-1126) or extracellular (aa 1039-1056) part of nephrin were generated (Harlow and Lane 1988). Briefly, these peptides were synthesized and purified, then the peptides were coupled to a multiple antigenic peptide-polylysine matrix and injected into two rabbits in Freund's complete adjuvant (Difco Laboratories, Detroit, MI, USA), and two booster immunizations 4 weeks after the previous immunization. Peptide-specific fractions were immunoaffinity-purified on CNBr-sepharose

(Pharmacia, Uppsala, Sweden) coupled to the corresponding linear peptides. The specificity of the antisera was tested by immunofluorescence on kidney sections with and without free peptide competition.

Development of monoclonal antibodies (IV)

Generation and characterization of mAb 18C7 (IgG2b) to isolated CNF glomeruli were described in detail elsewhere (Heikkilä *et al.*, in preparation). mAb 18C7 was produced according to standard protocols (Harlow and Lane 1988). Briefly, Balb/c mice were immunized with the antigen purified from one CNF kidney cells, and then the mice spleen cells were fused to myeloma cells. The clones thus obtained were subsequently subcloned. For glomerular positivity screening of the mAb, indirect IF on kidney sections was used. For initial charaterization of the antigenic epitope, treatment on tissue sections were performed using different reagents. Furthermore, immunoprecipitation and Western blotting were done.

Immunofluorescence techniques (I-IV)

Immunofluorescence techniques have been used for both tissue sections and cultured cells. Different fluorescein isothiocyanate (FITC)-labeled IgG was used in these experiments (DAKO, Glostrup, Denmark; Boehringer-Mannheim, Mannheim, Germany).

Immunohistochemistry and quantitative evaluation (IV)

An avidin-biotin technique was used, in which a biotinylated secondary antibody reacts with several peroxidase-conjugated streptavidin molecules. Briefly, after incubation with 0.5% avidin (Sigma Chimica, Gallarate, Milan, Italy) and 0.01% biotin (Sigma), tissue sections were fixed in a methanol-H_2O_2 solution. After washing, sections were sequentially incubated with the primary antibody, the secondary biotinylated antibody (Zymed, Histo-Line Laboratories, Milan, Italy) and the peroxidase-labeled streptavidin (Zymed). Peroxidase activity was detected with 3,5-diaminobenzidine (Sigma), then sections were counterstained with hematoxylin (DDK, Milan, Italy), dehydrated and mounted in Permount (DDK).

For the sections stained by 18C7 antibody, the following semi-quantitative score was applied: 0 = negative, 0.5 = focal and segmental positivity, 1 = diffuse segmental positivity, 2 = global and diffuse positivity.

Images were digitised using a videocamera (Kappa CF15/2, Gleichen, Germany) connected to a Leitz Diaplan microscope (Leica, Milan, Italy) and to a Pentium 120 computer (Maxwel, Rozzano, Italy) equipped with a frame grabber (Neotech Ltd, Easleigh Hampshire, UK). An automated macro composed by a color threshold procedure, filtering and Danielsson algorithm, was applied on all digitised images. Cell count was performed considering the number of positive cells per glomerulus (x 200), after drawing a precise line along the Bowman's capsule and programming the electronic system for ROI (region of interest) analysis.

Immuno-electron microscopy (I)

Postembedding electron microscopy was done, using CNF and normal cortical kidney samples fixed in freshly prepared 4% formaldehyde in PBS, then embedded in Lowicryl K4M (Chemische Werke LOW1, Waldkraiburg, Germany), and further incubated with the rabbit anti-nephrin antibodies and the respective gold conjugate.

Enzyme-linked immunoadsorbent assay (III)

ELISA was done in accordance with the method introduced by Kemeny (1991). Briefly, 100 ml of the respective intracellular or extracellular peptide was first bound to the 96-well microtiter plate (DNA-bind, Corning Costar Corp., MA, USA) for 2 hours. The optimal concentration (1 mg/ml) of peptide was selected after testing 100, 10, and 1 mg/ml respectively for coating. After thorough washing in PBS, 2% bovine serum albumin (Fraction V, Boehringer-Mannheim) in PBS was used for blocking overnight at +4°C. After thorough washing, 1:50 and 1:200 dilutions of patient sera in 10% fetal calf serum (FCS)-PBS were incubated for 2 hours. After washing, peroxidase-conjugated swine anti-rabbit IgG (1:2000 in 10% FCS-PBS; DAKO)

for 1 hour followed by 0.1 M citrate buffer (pH 5.0) containing o-phenylenediamine (0.4 mg/ml; DAKO) in 0.04% H_2O_2 and absorbance measured at 450 nm with an ELISA reader (Labsystems, Helsinki, Finland).

Western blotting (I, III, IV)

For immunoblotting, human kidney glomeruli were solubilized in RIPA buffer and centrifuged. The supernatants were run in reducing Laemmli buffer through polyacrylamide gels in Protean Mini-gel electrophoresis system (Bio-Rad Laboratories, Richmond, CA, USA). After electrophoresis proteins were transferred to and blocked on nitrocellulose filters (Schleicher & Schuell, Dassel, Germany), they were incubated with either nephrin pAb or mAb 18C7. After washing, the bound antibodies were detected with the ECL™ blotting kit (Amersham LifeScience, Amersham Int., Buckinghamshire, UK) according to the manufacturer's instructions.

Cell culture (II)

Adenovirus transformed human embryonic kidney cells, *i.e.* A293 cells (ATCC, Rockville, VA, USA), were used. They were cultured in RPMI medium (Gibco Biocult, Paisley, UK) containing 10% FCS, penicillin (100 IU/ml; NordCell, Skärholmen, Sweden) and streptomycin (100 µg/ml; NordCell). The other cell types tested included the HL-60, GEC, MDCK, NRK, and L2 cells. All cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA).

Cell stimulants (II)

For A293 cells, ocadaic acid, phorbol-12-myristate-13-acetate (PMA), lysophosphatidic acid, and bradykinin were used. For calcium signalling, Ca^{2+}, angiotensin II and arginine vasopressin were tested either respectively or in combination. They were all available from Sigma Chemical Corp. (St. Louis, MO, USA).

$[Ca^{2+}]_i$ measurement (II)

$[Ca^{2+}]_i$ was measured fluorometrically in CNF and normal human kidney cells (Haltia *et al.* 1997) loaded with the intracellular fura-2 (Molecular Probes, Eugene, OR, USA). Briefly, after withdrawing fetal bovine serum for 24 hours prior to the experiments, confluent monolayers grown on plastic Aclar coverslips (Applied Engineered Plastics, Potssville, PA, USA) were loaded with 1 µmol/l fura-2 in serum-free DMEM for 40 minutes at 37°C, followed by further incubation for 20 minutes in the same medium without the dye. Fluorescence measurements were performed by holding the coverslips diagonally in a quartz cuvette filled with 2 ml of modified Krebs-Henseleit solution. The monolayers were excited at 340 nm with emission collected at 500 nm in a Perkin-Elmer LS5B spectrofluorometer (Beaconsfield, UK). Excitation/emission slits were set at 2.5/5 nm, respectively. Standard formulae were employed for the calculation of $[Ca^{2+}]_i$, employing a K_d of fura-2 for Ca^{2+} of 224 nmol/l.

Mutation analysis (I)

DNA was isolated and then the respective exon areas were amplified by PCR using AmpliTaq DNA polymerase (Perkin-Elmer). The sequencing was done using the ABIPrism (Perkin-Elmer).

Statistics (II, IV)

Analysis of variance and χ-square test were used in Article II and IV, respectively.

RESULTS

Localization of nephrin in the glomerulus (I)

Immunofluorescence with polyclonal antibodies against the intracellular nephrin peptide was used to locate nephrin in normal glomerulus. In the kidney, nephrin was showed to exist exclusively in the glomerulus, moreover, the finely dotted linear reactivity giving a preferentially epithelial-like staining pattern could also be seen. No appreciable reactivity of the underlying GBM, endothelia, mesangium, and tubuli could be observed. Antibodies to the extracellular nephrin domain showed a closely similar glomerular reactivity pattern. Twenty seven of 28 CNF samples failed to show reactivity in glomeruli with the extra- and intracellular nephrin antibodies.

In immuno-electron microscopy, the anti-intracellular nephrin antibodies characteristically labeled the podocyte foot processes prominently at the filtration slit areas in normal kidney. However, some immunogold particles were also seen in the plasma membrane of podocytes, preferentially in the vicinity of the filtration slits but also in clusters at the apical surface (**Fig. 4**). In CNF kidney samples, some nephrin-specific gold particles were seen at the flattened apical surface of podocytes in association with microvilli and rarely at the intercellular junctional areas.

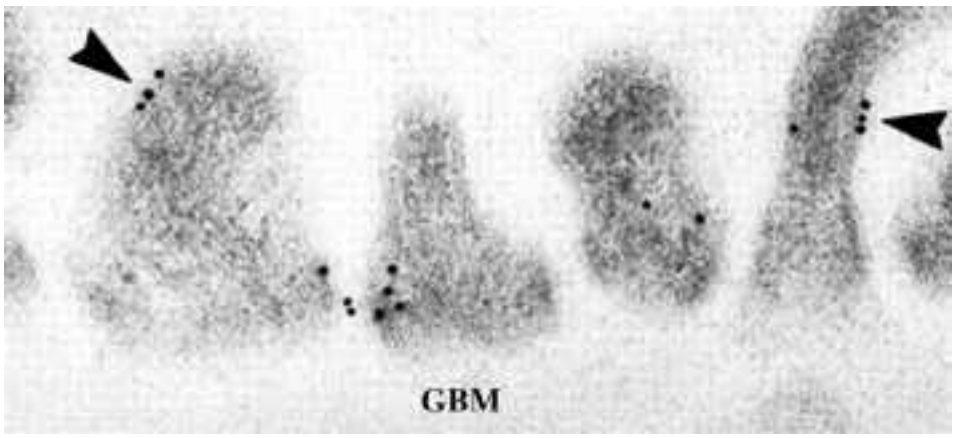

Figure 4. Localization of nephrin in normal human glomerular epithelium

Kidney nephrin expression in normal controls and CNF patients (I)

Northern blotting with cortical kidney failed to show clear reactivity of nephrin mRNA. Thus, a systemic RT-PCR analysis from normal human and CNF kidneys was performed with primers flanking the transmembrane domain. Two PCR products were seen, *i.e.*, the dominant band with the expected size and a second PCR product, designated nephrin-α with a calculated M_r 131 kDa. The sequence of nephrin-α is identical with that of nephrin except that nucleotides

3167-3286 of exon 24 are missing. This exon includes the putative transmembrane region of nephrin (nucleotides 3178-3258).

Of twenty eight CNF kidney samples, one showed nephrin antibody reactivity within glomeruli. However, mutation analysis by direct sequencing of exon 2 (Fin_{major}) and exon 26 (Fin_{minor}) was negative for both Fin_{major} and Fin_{minor} mutations.

Nephrin upregulation by PKC (II)

A293 epithelial cells of human fetal kidney were found to express both nephrin-specific mRNA and the respective protein (Luimula *et al.*, in preparation). Thus, this cell line was selected for further detailed study. Immunostaining for nephrin before and after the use of different stimulants revealed no obvious changes in staining intensity after lysophosphatidic acid, ocadaic acid or bradykinin treatments. However, consistently after PMA (PKC activator) stimulation, the augment of staining intensity was observed.

Table 4 Quantitation of nephrin mRNA in cultured A293 cells

Stimulants	24 hours	48 hours
Control	1.00 ± 0.28	1.00 ± 0.24
+ Ocadaic acid 1 nM	1.18 ± 0.17	1.25 ± 0.21
+ PMA 100 nM	1.40 ± 0.27	3.40 ± 0.34*
+ Lysophosphatidic acid 58 nM	0.95 ± 0.14	0.82 ± 0.17
+ Bradykinin 1 μM	0.98 ± 0.22	1.07 ± 0.24

Data are expressed as mean ± SE. * $P < 0.01$ (n = 3)

With the semiquantitative PCR, upregulation of nephrin-specific mRNA was readily observed on PMA by up to 340%. In contrast, ocadaic acid and bradykinin showed negligible upregulation of nephrin by 125% and 107%, respectively as compared to the level of β-actin, while lysophosphatidic acid decreased the mRNA level of nephrin to 82%. In the time-course experiment using PMA stimulation, no appreciable changes were seen at 2, 4, 8, or 12 hours, whereas at 24 hours nephrin-specific mRNA started to increase and was at a maximum at 48 hours (**Table 4**).

Baseline $[Ca^{2+}]_i$ in CNF and normal human kidney cells equilibrated in nominally Ca^{2+}-free media was 74.6 ± 8.4 and 69.4 ± 4.1 respectively. Upon graded addition of extracellular Ca^{2+}, $[Ca^{2+}]_i$ increased in both cell populations. No statistically significant differences were observed between the two groups. With angiotensin II and arginine vasopressin stimulation, both CNF and normal cells displayed a rapid elevation of $[Ca^{2+}]_i$, again, no significant difference in the timing and amplitude of responses was found between them. Consistent with the model of a store-activated, "capacitative" Ca^{2+} influx, angiotensin II stimulated Ca^{2+} entry upon addition of 1 or 10 mM extracellular Ca^{2+} in both cell lines, without appreciable differences. However,

when peak responses were factored for baseline $[Ca^{2+}]_i$, a reduced amplitude of the $[Ca^{2+}]_i$ changes in response to application of extracellular Ca^{2+} and/or vasoconstrictors was noted in CNF cells (*e.g.*, Ca^{2+} 1mM, CNF cells +117% vs normal cells +186%; + angiotensin II, CNF +767% vs normal +1028%; Ca^{2+} 1mM + angiotensin II, CNF +280% vs normal +408%).

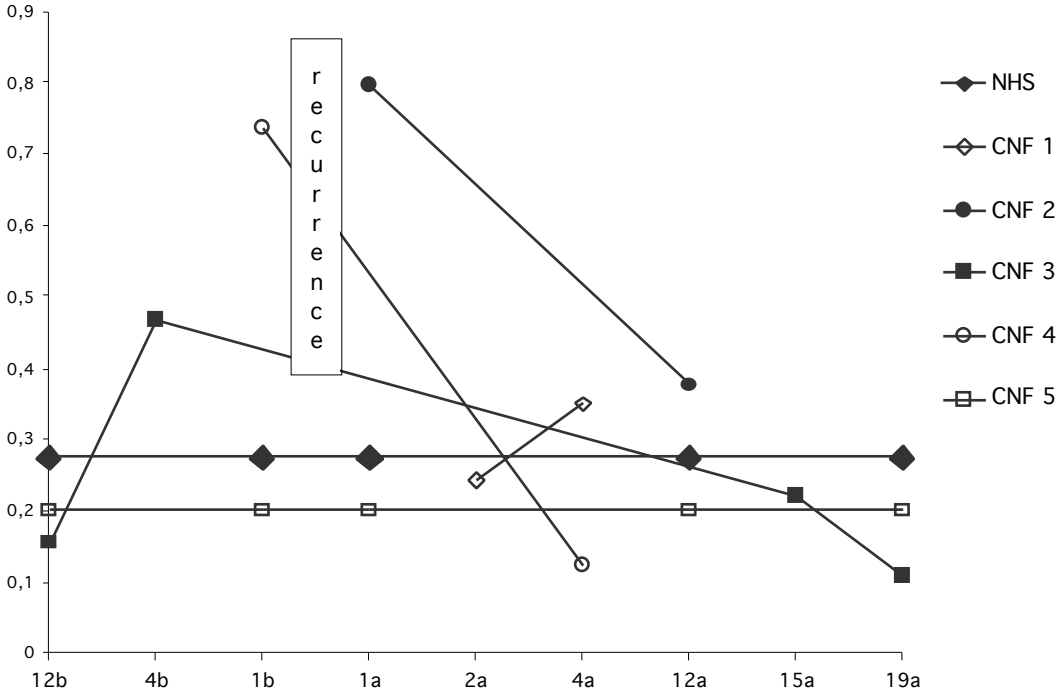

Figure 5. Five CNF patient sera before and after recurrence of the nephrotic syndrome together with normal human sera (NHS). X- and Y-axis represent months and absorbance at 450 nm (b, before recurrence; a, after recurrence)

Autoantibodies to nephrin in transplanted CNF patients with recurrence of nephrotic syndrome (III)

In CNF patients with recurrence of nephrotic syndrome, there was no over-representation in donor source, acute rejection or septic infections or significant HLA-A and -B mismatches, and blood cyclosporine concentration was within target limits. Serum creatinine concentration had increased slightly since the previous hospital visit. Serum albumin and protein concentrations were characteristically low and all patients had heavy proteinuria.

When the serum from one patient with a high titer of autoantibodies to nephrin was used to stain normal sections of human kidney, a faint and patchy glomerular reactivity was seen.

Optimization of the ELISA assay was achieved by using different concentrations of the coating peptide and by preincubation of the patient serum with the competitive oligopeptides, respectively. Also the controls of the second and irrelevant antibody reactivities with or without

coating peptide were negative. After successful treatment of the recurrence episode with steroids, cyclophosphamide and cyclosporine, the antibody titres of the individual patients decreased within 1-3 months for both the intracellular and extracellular antibodies (**Fig. 5**).

Table 5 Comparison of clinical and histological features according to 18C7 antigen expression

18C7 staining grade	Number	Urinary protein (g/day)	GBM thickness
0	59	2.3 ± 4.7	0.2 ± 0.5
0.5	28	3.6 ± 3.4	0.4 ± 0.6
1	16	2.9 ± 2.3	0.5 ± 0.7$^{\&}$
2	17	5.6 ± 3.0$^{*\#}$	1.6 ± 0.5$^{**\#\#\S}$

Data are expressed as mean ± SD. & $P = 0.05$ 0 vs 1; * $P < 0.05$, ** < 0.001, 0 vs 2; § $P < 0.001$, 0.5 vs 2; # $P < 0.05$, ## < 0.001, 1 vs 2

Expression patterns of nephrin and 18C7 antigen in human renal diseases (IV)

Monoclonal antibodies obtained were screened with tissue sections of CNF and the respective normal human kidneys (Ahola *et al.*, in preparation). One clone (18C7) was prominently specific for CNF while no reactivity of normal human kidney tissue was observed.

In further characterization of the 18C7 epitope, the tissue pretreatments with proteolytic digestion removed all tissue reactivity while the pretreatments for lipids did not have an effect, showing that the epitope is a protein. No reactivity in normal rabbit, mouse, and rat kidneys, fetal rat kidney, and human kidney were observed. In Western blotting of glomerular lysates, a single distinct band at 240 kDa was seen.

The staining for all normal human kidneys was negative for mAb 18C7, while the pAbs to extra- and intracellular nephrin domains gave their typical decoration in a podocyte fashion.

Among 120 kidney biopsies (**Table 3**) stained with anti-nephrin antibodies, no remarkable quantitative changes were detected with the antibodies to extra- or intra-cellular domains at light microscopy. With mAb 18C7, 59 samples were completely negative within glomeruli. Glomerular positivity, instead, was found in 61 of the biopsies: the staining was always visceral with different degrees of intensity and diffusion (**Fig. 6**).

A moderate (++) glomerular positivity for mAb 18C7 was detected in samples with MGN, MPGN, systemic lupus erythematosus (class IV), and cryoglobulinemic nephritis (5, 1.7, 5, 1.7% of the diagnostic group, respectively) (**Fig. 6**).

Among the 40 cases of primary and secondary IgA nephropathy, twenty-eight biopsies were negative and among the 12 positive cases, none showed a moderate positivity (**Fig. 6**).

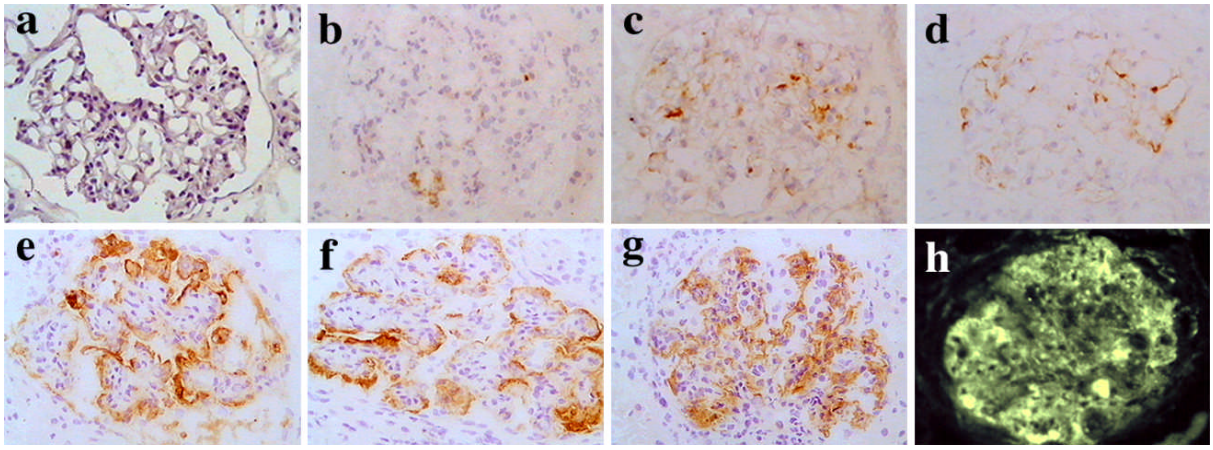

Figure 6. Immunoperoxidase (a-g) and immunofluorescence (h) staining of different diagnostic kidney biopsies by 18C7. a, normal human kidney; b, IgA nephropathy; c, MGN; d, FSGS; e, cryoglobulinemic glomerulonephritis; f, MPGN; g, systemic lupus erythematosus; h, CNF. original magnification, x 200

In addition to the association with the increasing trend in proteinuria (**Table 5**), global and segmental glomerular sclerosis and mesangial proliferation accompanied the different levels of positivity by 18C7, no statistical difference could be found among the groups. Instead, the statistical analysis disclosed a significant association between 18C7 positivity and the GBM thickness (**Table 5**). In 13 cases with different degrees of glomerular positivity, some endothelial staining was detected, mostly localized at the vascular pole of the glomerulus, but also present in some interstitial small sized vessels. When biopsies were categorised according to the presence or absence of endothelial staining, no statistical significance was obtained for any clinical and histological parameters.

DISCUSSION

Nephrin, a putative transmembrane protein, was first reported by Kestilä *et al.* (1998). It consists of an extracellular domain containing eight Ig-like modules and one fibronectin type III-like module, a single transmembrane domain and a cytosolic domain containing nine tyrosines. The mutations of NPHS1 (Fin_{major} and Fin_{minor}) are causative of over 90% of CNF cases. CNF is characterized mainly by massive proteinuria and considered to be a unique human single gene model disease of the perturbed glomerular filtration function (Haltia *et al.* 1997, Kestilä *et al.* 1998, Holthöfer *et al.* 1999a&b). Northern blotting showed that nephrin expression was restricted mainly to the kidney. However, very recently it was also found to be expressed in some parts of the mouse brain (Putaala *et al.* 2000). *In situ* hybridization showed that within the kidney nephrin exists exclusively in the glomerular epithelial podocytes (Kestilä *et al.* 1998). Further detailed *in vitro and in vivo* functions, however, are not clear.

Localization of nephrin in normal human glomerulus

Firstly, we started to study the exact location of nephrin by immunofluorescence and immuno-electron microscopy. In normal human kidney tissues, nephrin was detected uniquely in the glomeruli by using polyclonal antibodies against intra- or extracellular nephrin-specific domains. Staining by these antibodies demonstrated a typical glomerular podocyte pattern reactivity. Immuno-electron microscopy revealed a distinct localization of nephrin at the filtration slit areas of the podocytes (**I**). This discovery of nephrin location at the slit diaphragm provides a strong support for the hypothesis raised by Kestilä *et al.* (1998). It was hypothesised that the homophilic interactions of six extracellular modules of nephrin constitute the zipper-like structure between foot processes, preventing plasma proteins, especially albumin from leaking. Ig repeats from 1 to 6 of a nephrin molecule from one foot process associate in an interdigitating way with the same Ig repeats 1-6 protruding out from the opposite foot process of another podocyte. In addition, disulfide bonds are formed between different cysteine residues. The cysteine of the fibronectin domain may interact with neighbouring nephrin molecules or others (Kestilä *et al.* 1998, Tryggvason *et al.* 1999, Wickelgren 1999, Ruotsalainen et al. 1999).

However, recent evidence indicates that nephrin may not be the only molecule at the slit pore; other proteins, *i.e.* P-cadherin and podocin, probably participate in the maintenance of the pore structure between foot processes (Reiser *et al.* 2000).

Based upon the previous main finding that ZO-1 associated with the cytoskeleton at the slit diaphragm, it was thought that the slit diaphragm represented a modified tight junction (Kurihara *et al.* 1992). Recently, P-cadherin, CD2AP, α-, β-, γ-catenins, α-actinin-4, and vinculin were also located at the cellular junctions of the podocytes *in vitro* (Shih *et al.* 1999, Reiser *et al.* 2000, Somlo and Mundel 2000). However, no any reports were found on other TJ-

associated molecules (occludin, claudin, ZO-2, 7H6, cingulin) at the slit diaphragm (Bains *et al.* 1997, Reiser *et al.* 2000). In addition, desmoglein and desmocollin were not detected in cultured podocytes (Reiser *et al.* 2000). Therefore, Reiser *et al.* (2000) presented their hypothesis: P-cadherin stands out as the core protein in the assembly of the slit diaphragm. The intracellular part of P-cadherin is connected with different catenins, whereby α-actinin is connected directly to the cytoskeleton or is bridged by ZO-1 and α-actinin-4 between them (Reiser *et al.* 2000, Somlo and Mundel 2000). Thereby, the slit diaphragm appears a modified adherents junction rather than a tight junction as traditionally thought.

However, some disagreements still existed. Bains *et al.* (1997) failed to detect any cadherins and catenins either in normal human kidney biopsies or in those of proteinuric states. In addition, nine connexins were reported to be expressed in the kidney, but little was known of the detailed sublocalizations (Guo *et al.* 1998). No further articles describing connexins in podocytes were found (Simon and Goodenough 1998). Thereby, there will be a long way to go before the slit diaphragm is completely delineated.

Glomerular nephrin expression under immuno-EM in CNF patients

Nephrin was seen in immuno-EM analyses of some of the CNF samples studied, preferentially at the plasma membrane of the podocytes (**I**). This result appears controversial because, particularly in Fin_{major}, no nephrin protein should be expressed (Lenkkeri *et al.* 1999). However, only homozygosity in respect to Fin_{major} mutation leads to a complete lack of nephrin, whereas the Fin_{minor} mutation found in 20% of the Finnish patients causes a nonsense mutation at exon 26 in the intracellular domain. This site is downstream, beyond the recognition site of our intracellular antibodies and, if transcribed and translated, our antibodies should normally also detect this product. Thus, the heterozygotes may produce nephrin detectable only in immuno-EM analysis but not in indirect IF microscopy.

Identification and function of nephrin-α

When PCR was used to detect the nephrin mRNA, two bands were observed in normal human kidney (**I**). These PCR products were further cloned, sequenced, and verified to be nephrin-specific. Interestingly, the novel nephrin-α variant misses the whole aa sequence spanning the transmembrane domain (encoded by exon 24). The analysis of nephrin in normal human kidney by SDS-PAGE also identified two different bands, the dominant band and a slightly low band (Ahola *et al.* 1999). That such a splicing yields variants without the complete transmembrane area is interesting and could lead to secretion of the protein. Examples of such secreted transmembrane splicing variants, with biologically important regulation of the respective receptor affecting the ligand binding, include interleukin-6 (Säily *et al.* 1998) and T cell receptor (Takase *et al.* 1998). So far, the functions of nephrin-α are largely unknown. Whether and how

nephrin-α participates in the maintenance of the slit diaphragm need to be studied further. Luimula *et al.* (2000b) very recently reported the existence of nephrin protein in the urine, suggesting its important roles in proteinuric diseases.

New insights into the mechanisms of glomerular proteinuria in CNF patients

In CNF patients, as showed previously, no clear pathogenesis was known. However, the discovery of nephrin should prompt an in depth study of the pathogenesis of CNF. If the hypothesis raised by Kestilä *et al.* (1998) was right, the defective structural basis of the slit diaphragm would contribute totally to heavy proteinuria found in CNF patients. Indeed, the mechanisms appear far from so simple. For instance, Holthöfer *et al.* (1999b) found that the subunit I of cytochrome c oxidase was downregulated in kidneys from the CNF patients, being in parallel with the increase of lipid peroxidation in glomeruli. Overgeneration of ROS and accumulation of local lipid peroxidation adducts could give rise to glomerular proteinuria. Therefore, how nephrin is relevant to the changes of the mitochondria and lipid is still unknown. Based upon the data by Tryggvason (1977) and Risteli *et al.* (1982), glomerular collagen metabolism dysfunction might be one characteristic in CNF patients. How nephrin is associated with collagen metabolism needs to be studied further.

Nephrin upregulation by PKC

The knowledge of nephrin distribution appears to be the first step in elucidating its functional roles. A further question was what are the regulators of nephrin in the cell. Consequently, we set up a cell culture system to explore the pathways responsible for the regulation of nephrin.

Before starting this phase of the work, we tested several cell lines, for instance, HL-60, GEC, MDCK, NRK, L2, and A293. They were all found to express nephrin by use of the RT-PCR method. It was decided to use A293 cell line, based mainly upon its being a kidney epithelial-derived cell line and naturally expressing nephrin. In order to induce the possible changes in nephrin, several agents were chosen, including ocadaic acid, bradykinin, PMA, and lysophosphatidic acid. Interestingly, PMA was found to upregulate the expression of nephrin at the mRNA level. Concomitantly, at the protein level, we found only a slight change (**II**). It has been known that PMA can activate protein kinase C, which is one important source for phosphorylating other proteins.

Phosphorylation is an important step in activating proteins. One-third of all proteins in eukaryotic cells were thought to be phosphorylated at any one time (Zolnierowicz and Bollen 2000). PKC has been showed to be expressed widely in different tissues including the kidney (Mellor and Parker 1998, Newton and Johnson 1998, Ron and Kazanietz 1999). Of the eleven PKC members, PMA can activate cPKC (α, β1, β2, γ) and nPKC (δ, ε, η, θ) isoforms,

consequently, any isoforms of them (α, β1, β2, γ, δ, ε, η, θ) could be involved in phosphorylation in our experiment. The precise pattern of the isoforms can be determined in the future.

How the cell signalling is regulated from PKC to nephrin is really intriguing. Thereby, how many and which signalling pathways are responsible for nephrin regulation need to be studied further.

Little is known about the signalling pathway of PKC in intercellular junctions, but multiple lines of evidence have showed that PKC closely participates in the assembly, regulation, and communication of them. The intercellular junctions, *i.e.* tight junctions, adherents junctions, gap junctions, and desmosomes, all have been reported to be regulated by PKC whether its role is direct or indirect (Ratcliffe *et al.* 1997). Many putative targets of PKC, in association with intercellular junctions, have been identified, such as c-Raf (Kolch *et al.* 1993), vinculin (Perez-Moreno *et al.* 1998), talin (Litchtfield and Ball 1986), MARCKS (Aderem 1992), glycogen synthase kinase-3 (Goode *et al.* 1992), focal adhesion kinase (Zachary and Rozengurt 1992), p120/p100 (Ratcliffe *et al.* 1997), connexin43 (Lampe *et al.* 2000). In human keratinocytes, it was found that E-cadherin mediates adherents junction organization via PKC (Lewis *et al.* 1995). In MDCK cells, during the assembly of tight junctions, PKC plays a significant role, in association with TJ proteins, such as ZO-1 and cingulin (Stuart and Nigam 1995). The downregulation of the mitogen-activated protein kinase pathway is highly involved in the restoration of tight junction structure and barrier function (Chen *et al.* 2000). In LLC-PK1 cells, overexpression of C-δ was reported to increase the TJ permeability, while C-α to modulate transepithelial permeability and cell junctions (Rosson *et al.* 1997, Mullin *et al.* 1998). It was found that connexin43 on serine368 is phosphorylated by PKC and plays an important role in gap junction communication (Lampe *et al.* 2000). C-ε is needed to phosphorylate cardiomyocyte connexin43 (Doble *et al.* 2000). Finally, PKC is heavily involved in the adhesive regulation of desmosomes (Kitajima *et al.* 1999). We have not studied the function of nephrin in the assembly of cellular junctions, therefore, this could be one of our long-term goals.

In our experiment, G-protein-linked signalling pathways by lysophosphatidic acid (Moolenaar 1995) and bradykinin (Burch and Kyle 1992) and protein phosphotase 2A and 1 pathways by okadaic acid (a potent inhibitor) (Fujiki and Suganuma 1994) had no any effects on nephrin expression (**II**). In addition, the impact of nephrin deficiency in cultured CNF glomerular cells for Ca^{2+} signalling was examined. No significant changes were found in comparison with glomerular cells grown from normal individuals, irrespective of the stimuli applied. However, a trend towards a reduced amplitude of the $[Ca^{2+}]_i$ changes in response to application of extracellular Ca^{2+} and/or vasoconstrictors was observed when peak response were factored for resting $[Ca^{2+}]_i$ in CNF cells (**II**).

Autoantibodies to nephrin in transplanted CNF patients with recurrence of nephrotic syndrome

In CNF transplantation patients, 20% cases developed recurrence of nephrotic syndrome (Laine *et al.* 1993). However, the pathogenesis of recurrence is unclear. Here we showed that recurrence of nephrotic syndrome in CNF transplantation patients was associated with preceding high titers of specific antibodies to nephrin (**III**). The successful treatment of recurrence and subsequent maintenance with effective antirejection therapy resulted in a decrease in the antibody level within three to six months (**III**). This decrease followed a normal half-life kinetics of circulating antibodies. Nephrin-α is a major splicing variant of human nephrin and it exists in normal human kidney (**I**). Our ELISA method can not distinguish between nephrin and nephrin-α. Thus, the role of nephrin-α can not be ruled out in autoantibody production.

Basically, similar characteristics can be found in Alport syndrome patients with antibodies developed against GBM collagen IV (Brainwood *et al.* 1998). Thus, CNF appears to be the first human disease in which circulating antibodies were developed against podocyte epitopes. Prominently, the patients with recurrence of nephrotic syndrome regularly presented with a prior infection with Epstein-Barr or cytomegalovirus before the new recurrence episode. Whether this infection was the trigger by inducing a major generalized immunoactivation can only be speculated.

Whether autoantibodies to nephrin are the cause of the recurrence of nephrotic syndrome deserves to be studied deeply. Therefore, new experiments are needed to confirm the exact role of autoantibodies to nephrin.

It is clear that further study is needed to know whether there exists a relationship between recurrence and mutations of NPHS1, *i.e.*, Fin_{major} and Fin_{minor}, since not every patient had a high level of autoantibody titers to nephrin at the peak of recurrence.

Kidney nephrin expression in non-CNF human renal diseases

The characteristic nephrin mutations in the massively proteinuric CNF patients, induction of rapid proteinuria by *in vivo* injection of anti-nephrin antibodies (Topham *et al.* 1999) and remarkable downregulation of the nephrin-specific mRNA in experimental models (Luimula *et al.* 2000a, Kawachi *et al.* 2000) all suggest its crucial roles in the maintenance of the glomerular filter. Hence, we used anti-nephrin antibodies to evaluate nephrin protein involvement in glomerular permeability changes in different diagnostic groups of human renal diseases (**Table 3**). No appreciable qualitative and minor quantitative changes were seen when antibodies to nephrin were used (**IV**). This seems in contradiction to the results reported by Furness *et al.* (1999). They performed PCR amplification of nephrin cDNA and found that glomerular levels of nephrin mRNA was decreased in several MCGN and MGN cases. The detailed reason for such a difference needs to be studied further.

Kidney 18C7 antigen expression in non-CNF human renal diseases

mAb 18C7, normally failing to react with normal glomeruli, was used to stain the human kidney sections of the renal diseases. Strong reactivity patterns were seen in CNF and some other proteinuric kidney diseases (MGN, MPGN, systemic lupus erythematosus and cryoglobulinemic nephritis) (**IV**). Similar deposition of proteins such as dystroglycan (Luimula *et al.* 2000a) and apoE (Sato *et al.* 1991) was also found at these sites. Thus, whether these proteins are associated with the 18C7 epitope is currently unknown. In Western blotting of glomerular lysates in denaturing conditions, mAb 18C7 showed characteristic reactivity of an estimated 240 kDa. In addition, radial immunodiffusion method was taken to determine whether 18C7 antigen was from the plasma. However, no positive results were obtained. Based on the evidence available, we considered that mAb 18C7 recognizes a glomerular epitope characteristically modified or expressed in proteinuria.

Of the various layers of the glomerular filtration barrier, GBM lies between the endothelium and the podocyte layers. GBM is supposed to play a molecular scaffold, mainly consisting of collagen IV, laminin, heparan proteoglycan and entactin/nidogen (Tryggvason 1999, Kanwar *et al.* 1991, Miner 1999, Hudson *et al.* 1993, Caulfield and Farquhar 1976), produced by all glomerular cell types, including podocytes. Genetic mutation of GBM components resulted in disorders of the permeability as well, thus reflecting alterations in the respective glomerular cell type. Here we found a strong association of 18C7 protein accumulation with GBM thickening, which may directly reflect alterations of the podocyte metabolism in diseases. But obviously, more work is needed in the future.

CONCLUSIONS

This series of studies on nephrin have touched several issues, from localization of nephrin to its clinical associations. Firstly, nephrin is a new protein of the interpodocyte filtration slit area. The localization of nephrin at the slit diaphragm is the first step in elucidating its functions in the maintenance of glomerular filtration barrier. Secondly, the activation of protein kinase C upregulates the expression of nephrin mRNA, which is an important indication for further biochemical or cellular studies. Thirdly, the autoantibodies to nephrin were found in CNF patients with recurrence of nephrotic syndrome. Finally, nephrin expression didn't differ in a variety of human renal diseases. 18C7 antigen, which was expressed in CNF patient kidney tissue but not in normal human kidney tissue, was strongly positive in the groups with heavy proteinuria. These suggest that many more molecules participate in the regulation of the glomerular filtration barrier.

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